VOL. 21, NOS. 3 AND 4 AUGUST 1945

THE SICKLING PHENOMENON AND ITS BEARING ON THE PROBLEM OF RED CELL STRUCTURE

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(*Received* 25 *August* 1944)

(With One Text-figure)

In considering the various forms of disk-sphere transformation which mammalian red cells undergo, reference has repeatedly been made to the ideas of Norris, Gough, Teitel-Bernard and others who have suggested that the discoidal form of the cell is the result of an equilibrium between two sets of forces, one expansive, and the other (which includes surface tension) conducing to contraction of the surface (Norris, 1882; Gough, 1924; Teitel-Bernard, 1932; Ponder, 1929, 1934, 1936, 1937, 1939, 1941, 1942 *a*, *b*, *c*; Furchgott, 1940*a*, 1940*b*; Furchgott & Ponder, 1940, 1941). When the disk changes into the sphere, we are dealing with a diminution or disappearance of the 'expansive force' which is probably due to a modification or even a destruction of a surface ultrastructure, and in the absence of anything to oppose it, the tension at the cell-fluid interface determines that the cell takes up the spherical form in which the surface is a minimum for the volume. As will be seen below, other forces are probably operative in the same direction. A re-establishment of the ultrastructure, with the 'expansive forces' inherent in its molecular arrangement, leads to the transformation of the sphere into the original disk, and the various conditions under which this reversal can be brought about have already been described. We have now to consider a situation in which the 'expansive forces' are so great, or the forces resisting them so small, that the red cell assumes a series of shapes which are characterized by extension, just as the spherical form is characterized by contraction.

The reversible phenomenon of sickling, in which mammalian red cells in sealed wet preparations lose their biconcave discoidal form and become shaped like crescent moons or sickles, occurs in man in the pathological condition of *sickle-cell anaemia*, and also, without associated pathology, as the *sickle-cell trait*. The first of these conditions is familial (Mendelian dominant), and is only one of several degenerative stigmata (skeletal malformations, endocrine deficiencies, etc.) with which the individual is affected. The difference between sickle-cell anaemia and the sickle-cell trait is probably only one of degree. In the latter condition, sickles are not seen in stained films, but only in sealed wet preparations, and both the number of cells which become sickle-shaped and the rapidity of the deformation are less than in sickle-cell anaemia proper. O'Roke (1936) has described sickling of red cells in deer, and I have observed a similar phenomenon in the blood of elks.

I. THE SICKLING PHENOMENON AND ITS REVERSAL

The sickling phenomenon has often been described,* but not from the standpoint of its bearing on red cell structure; the existing descriptions accordingly fail to mention details which are of considerable interest from this point of view, and do not enable one to form a clear picture of what actually happens. Part of the difficulty lies in the fact that one cannot define the shape of the sickle cells adequately unless one can observe them and measure them as solid bodies in three dimensions rather than as objects seen only in the plane of the slide.

Observations and measurements. Using heparin as an anticoagulant, blood is obtained from an arm vein of a person with sickle-cell anaemia or the sickle-cell trait, and can be kept for several days in the refrigerator if necessary. Some of the plasma is separated and used to dilute the blood about 1 to 5, so that the cells will not be too closely packed together in the wet preparation. The wet preparation is made by putting a small drop of the diluted blood on a slide and covering it with a no. 0 cover-glass which has been vaselined along the edges. The thickness of the vaseline strips can be used to regulate the depth of the layer of plasma and cells. Nearly all my observations were made at 22°C.†

* Wintrobe (1942) gives an extensive bibliography (forty-five references) of papers on sickle-cell anaemia and the sickle-cell trait.

† Sickling proceeds more rapidly at higher temperatures, and is almost completely inhibited in the cold (4°C.). This may be partly due to the reduction in red and white cell metabolism at low temperatures, so that the O_2 tension falls more slowly, and partly to the viscosity and rigidity of the red cells being increased.

The cells are examined either with a high dry or with an oil-immersion objective and a 5*X* eyepiece, which can be replaced, for purposes of measurement, with a 10*X* filar micrometer. The complete determination of cell shape is possible only if the cells can be observed in a variety of positions and can be measured both on the flat and on edge. To do this, the individual cell under observation is moved about and oriented on edge by pressing on the cover-glass with a dissecting needle. With practice, one can acquire great skill in this form of manipulation. The needle, which should be about 2 in. long, not too flexible, and mounted in a handle, is placed with its tip quite close to the area of the cover-glass which is covered by the objective, and the cover-glass is pressed on firmly, the cells being kept in focus all the time. Variations in this firm pressure, which is continually maintained, cause the cells to orient themselves in different ways, and the individual cell can be turned up on edge for inspection, dropped back again into the plane of the slide, and so on. The ease with which this can be done depends largely on the vaseline seal being made so that only a thin layer (50–100 μ) is enclosed between slide and cover-glass; the less complete the seal, the more difficult is the manipulation of the cells, and the more readily are they carried about the field by random currents.

Nomenclature. The red cell which ultimately undergoes sickling is a biconcave disk undistinguishable in appearance from the normal erythrocyte; the normal red cell, however, retains its shape indefinitely, whereas the red cell in sickle-cell anaemia and the sickle-cell trait becomes a sickle cell or meniscocyte when the O_2 tension is reduced. I shall, therefore, refer to the discoidal form which becomes a meniscocyte as a promeniscocyte, and this cell will be morphologically indistinguishable from the normal erythrocyte.

The sickling phenomena. The phenomena of sickling is primarily due to the O_2 tension in the sealed preparation falling as a result of red and white cell metabolism (Hahn & Gillespie, 1927; Hahn, 1928; Sherman, 1940). After a period of time which varies with the thickness of the preparation, the temperature, and other factors, the promeniscocyte displays a change in rigidity which can be appreciated best by the manipulation of the cells as described above. The plasticity of the cell becomes greatly increased, so that slight forces in the fluid produce gross, rounded deformities of shape, such as one associates with droplets of an oil of low viscosity. At the same time, if the cell is turned up on edge, the upper part tends to fall over as if the cell were unable to stand up under its own weight. A normal erythrocyte has sufficient rigidity to stand on end like a piece of cardboard, but a promeniscocyte in the pre-sickling stage bends over in the middle like a piece of paper. When currents in the fluid bring promeniscocytes into collision with one another, the same lack of rigidity in the pre-sickling stage is shown by the amount of deformation which occurs; one cell will bend almost at right angles in flowing over a rounded projection on the other.

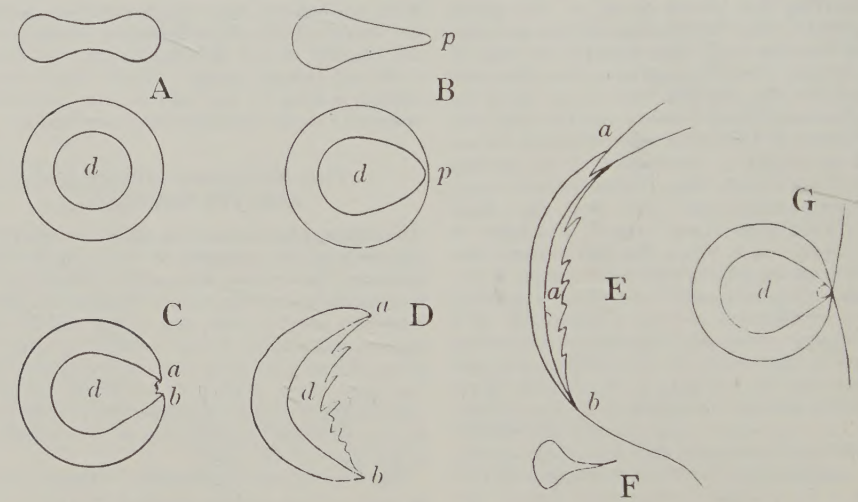

Fig. 1.

This change in rigidity is followed by the sickling phenomenon proper. The rim of the cell becomes thicker on one side and thinner on the opposite side (Fig. 1 A, B, which show the cell diagrammatically on the flat and in cross-section). This thinning pro-

ceeds until continuity breaks down at the point *p*; the two newly formed ends, *a* and *b*, are then separated by a straightening out of the arc *ab*, which stretches the material of the biconcavity between them as the curvature of the arc decreases (Fig. 1 C, D). The thinned and stretched material of the biconcavity expands at its unsupported edge into a series of veil-like projections (irregular lines in Fig. 1 D); meantime the straightening out of the arc continues, but is accompanied by a contraction along its length, so that the distance *ab* is finally much less than the perimeter of the original disk. The veil-like material of the biconcavity follows the change of form of the arc, and in the completed meniscocyte (Fig. 1 E) appears as a very thin and almost invisible fringe lying within the sickle. From measurements such as those given in Table 1 we can assign the form shown in Fig. 1 F to the cross-section of the typical meniscocyte.

The dimensions of the average cell before and after sickling are shown in Table 1, with all measurements in μ. The principal points to be observed are (*a*) that the volume does not change, (*b*) that the mean thickness increases, and (*c*) that the length of the arc *ab* decreases as the meniscocyte forms.

Table 1

Fig. 1 A	Diameter	9·0
	Mean thickness	1·3
	Volume, μ^3	83·0
Fig. 1 D	Distance *ab*	7·0
	Arc *ab*	28·0
	*Projected area, μ^2	55·0
	Mean thickness	1·5
	Volume, μ^3	83·0
Fig. 1 E	Distance *ab*	12·7
	Arc *ab*	20·5
	Distance *ef*	3·6
	*Projected area, μ^2	46·0
	Mean thickness	1·9
	Volume, μ^3	83·0

* This is the projected area of the 'body' of the meniscocyte, not including the area of filaments or of the material of the biconcavity.

The first stage of meniscocyte formation, in which one edge of the cell thickens while the other thins, may take one or two minutes, but once stage C is reached the remainder of the transformation takes place in about a second. It is very difficult to follow because it is so rapid. Once the sickle cell is formed, no further shape changes take place for hours or even days. Ultimately the cells round up, become prolytic spheres, and haemolyse.

To this description of the shape changes undergone by the body of the cell there must be added a description of the remarkably extensive filament formation which accompanies them. As the points *a* and *b* separate, each becomes extended outwards into a slender filament, 10μ or more in length. Sometimes there are two or more points corresponding to *a* or *b*, and in this case filaments may extend from each (Fig. 1 E). The scolloped edge of the material of the biconcavity may extend outwards into filaments, usually much shorter than those at the ends of the arc. These filaments are probably composed of lipoid derived from the cell envelope, and resemble those seen during the disintegration of ghosts (Furchgott, 1940). If the sickle cell is retransformed into the discoidal form, these long filaments break off at the base, and contract to form rods or globules which can be seen floating freely in the suspension medium.

While Fig. 1 is a diagrammatic representation of the sequence of events which constitute the promeniscocyte-meniscocyte transformation, the changes which occur in individual cells are subject to considerable amount of variation. Often the preliminary thickening occurs at two or even three places along the rim, so that very bizarre forms result when the meniscocyte is finally formed. Sherman (1940) has described a 'holly wreath' form of the sickle cell in which the haemoglobin seems to be concentrated at multiple foci along the rim of the cell, and which may be a combination of sickling with crenation.

The reversal of sickling. This occurs when the O_2 tension in the preparation is increased, and in preparations between slide and cover-glass can be brought about by running oxygenated plasma or saline between the glass surfaces through two small gaps in the vaseline seal. The events which occur are those of the sickling phenomenon in reverse. The arc *ab* becomes both longer and more curved, and the material of the biconcavity follows the changes of curvature so as to fill in the concavity just as a frog's web fills in the region between his toes. The free edge of this material loses its irregular projections, some by breaking off, some by retraction, and becomes smooth. The whole cell at this stage appears to be under tension. The curvature increases until it is almost that of a circle, the points *a* and *b* coming nearer to each other, and the material of the biconcavity filling up the interior of the ring more and more completely. The points *a* and *b* now touch, and the discoidal form is re-established, except for the fact that a small globule, which remains in the cell as a refractile body, is either formed or enclosed at the point of contact of *a* and *b*. If *a* and *b* have filaments attached to them, these can often be seen in the reconstituted cell lying tangentially to the refractile globule at the point of contact (Fig. 1 G). After a time they disappear. The rate of the reversal process depends on the rate at which the O_2 tension is re-established in the preparation. Under suitable conditions it takes some minutes to complete, and the intermediate stages can easily be observed. If O_2 is admitted suddenly, as by lifting the cover-glass of a sealed preparation, the reversal of sickling is as rapid as sickling itself.

While the refractile body is formed at the point where the two ends of the sickle cell fuse in the com-

pletion of the restoration of the discoidal form, it does not necessarily mark the spot at which the ring will open up if sickling is re-induced. The thinning of the rim of the cell which precedes the beginning of sickle formation may apparently take place at any place on the circumference, and the point at which the material of the rim is drawn out into the two points *a* and *b* is not necessarily near the position of the refractile body. This disposes of the simple idea that the material of the rim is drawn out at an intrinsically weak spot produced by some event such as previous sickle formation or the extrusion of the normoblast nucleus.

II. DISK-SPHERE TRANSFORMATIONS IN PROMENISCOCYTES AND MENISCOCYTES

The remarkable disk-sphere transformations which can be brought about by lecithin, rose bengal, saponin, and other agents have already thrown considerable light on the structure of the red cells of mammals and the lower vertebrates, and so we shall enquire whether the same or similar shape transformations occur in (*a*) the discoidal promeniscocytes, and (*b*) the sickle cells themselves.

The disk-sphere transformations which occur in promeniscocytes can be studied between plastic surfaces exactly as the shape changes of other mammalian red cells are studied. The experiments are best carried out with suspensions of washed cells, and it will be found that the shape changes are indistinguishable from those observed with normal human erythrocytes. Lecithin sols and rose bengal in a concentration of about $10^{-5}M$ cause the cells to become spheres which revert to disks again on the addition of plasma. If the action of the lecithin or of the dye is sufficiently prolonged, the cells haemolyse as spheres. Saponin and other lysins cause the cells to crenate, to become prolytic spheres, and finally to haemolyse; the ghosts are roughly spherical. Between *glass* surfaces, spherical forms are produced as a result of the increase in *p*H together with the removal of anti-sphering substance from the cell surfaces; disks are immediately reformed if plasma is added.*

The study of the shape changes which occur in sickle cells on the addition of lecithin, rose bengal, etc., is very much more difficult, for the sickle cell occurs as a table form only at a low O_2 tension, and the addition to a slide and cover-glass preparation of a substance in solution (e.g. lecithin or a dye) leaves one unable to say with certainty that the effects observed are due solely to the added substance and not to O_2 carried in with the solvent. This difficulty is best surmounted by using Hahn's method of studying the meniscocytes in a hanging drop in a chamber from which the O_2 can be replaced by CO_2 or N_2.

A simple chamber can be made from a 25 mm. square block of paraffin wax, about 15 mm. high, through which is cut a circular hole about 15 mm. in diameter. This makes a chamber, the top and bottom open, which is stuck on to a 3 in. × 1 in. glass slide. An entrance tube and an exit tube are added by pushing warmed glass tubing through opposite sides near the bottom of the chamber; they seal themselves in as they cool. The glass slide carrying the chamber is held in the moving stage of the microscope, and the preparation is in the form of a hanging drop on a cover-glass which is sealed on to the top of the chamber with thin vaseline. A high dry objective is used to examine the cells. Gas from a CO_2 tank is led into the chamber after passing through wash bottles and a water blow-off, so that it is supplied in a constant stream at a pressure of about 60 mm. of water. While the gas is flowing into the chamber the cells of the hanging drop are usually in motion and cannot be seen distinctly, but if the flow is cut off momentarily by pinching the inlet tube they become stationary almost instantly, and their shape can be observed. A small aperture cut out of the upper edge of the chamber on the side nearest the observer allows lysins, etc., to be added to the hanging drop. O_2 must be removed from the saline in which the substances to be added are dissolved; alternatively, lysins such as saponin can be added at one edge of the hanging drop in the solid state, and allowed to reach the cells by diffusion.

The meniscocytes which are formed as O_2 is displaced from the chamber undergo no sudden change of shape on the addition of rose bengal, lecithin, or saponin. They remain as sickles until the time of lysis, when they turn into spheres by a process very similar to the reversal of sickling described above, and fade from view. In some cases the fading occurs first, and the sickle becomes a sphere shortly after it has lost its pigment; in other cases the shape change occurs first, and the fading soon follows. The ghosts are never sickle-shaped, but are always irregularly spherical. This last observation is not surprising, for the ghosts of normal human red cells which have been haemolysed with saponin and most other lysins are substantially spherical, although often irregularly crenated masses.

The ghosts obtained by haemolysing either promeniscocytes or meniscocytes by water, however, are discoidal, in conformity with the rule that mammalian watery ghosts are biconcave disks, although often showing considerable crenation. Exposure of meniscocyte ghosts to low O_2 tensions does not result in any shape change of the sickling type, since the

* Through the kindness of Dr E. J. Cohn, Dr B. H. Vickery, and Dr Hans Neurath, I have been able to obtain a number of plasma protein fractions in a state of known purity, and have been able to confirm the conclusion (Furchgott & Ponder, 1940) that only the albumin fraction produces reversal of the slide-cover-glass shape transformation. Cholesterol, in the form of the sol prepared by the method of Lee & Tsai (1942), does not reverse the disk-sphere transformation produced by lecithin.

presence of haemoglobin in the cell is necessary for the disk sickle-cell transformation.

III. THE BEARING OF THE SICKLING PHENOMENON ON THE PROBLEM OF RED CELL STRUCTURE

The disk-sphere transformations of the mammalian red cell can all be accounted for in terms of a surface ultrastructure which has a discoidal biconcave form because of the nature, orientation, and repulsive forces between its component molecules. It has already been suggested that the forces which maintain the surface as a biconcave disk are repulsions between the side chains of radially arranged cephalin molecules; whether this be so or not, the forces which keep the surface in its expanded state can be reduced by various means (addition of lecithin, action of rose bengal, removal of anti-sphering substance, etc.), under which circumstances the disk becomes the body of smallest surface for its volume, the sphere. Other operations (washing off of lecithin or rose bengal, addition of anti-sphering substance, etc.) result in the original forces being re-established in the ultrastructure, and in a conversion of the sphere into the disk. We have now to extend our conception of red cell structure so as to account for the shape changes involved in sickle cell formation and its reversal. To do this we have to suppose that the haemoglobin in the cell exerts an 'expansive force', a function of the O_2 tension, which in the normal erythrocyte produces only tension or turgor because the surface ultrastructure is intact, but which produces sickling in the promeniscocyte and meniscocyte because the surface ultrastructure is defective. Thus we have three types of force to consider instead of the original two, and they are:

Type I. The forces of surface tension, which tend to produce the spherical form. It should be remembered that there are two surfaces, one outside and one inside the ultrastructure.

Type II. The intermolecular forces in the surface ultrastructure, which tend to maintain it in a biconcave discoidal form and with a surface in excess of the smallest surface for the enclosed volume, and

Type III. The forces existing in the red cell interior, and particularly associated with the haemoglobin, which are 'expansive' or 'turgor-producing' and which tend to force the enveloping ultrastructure into the various forms assumed by the meniscocyte.*

These forces may be thought of as small (but not absent) when the haemoglobin is fully oxygenated, and as becoming much greater when the O_2 tension falls towards zero.*

With these three forces acting upon it, the normal biconcave discoidal form can be regarded as one of three metastable states, and the rigidity of its ultrastructure is sufficient to resist deformation by either the force of surface tension or the turgor-producing forces associated with oxyhaemoglobin. If the turgor-producing forces are strong enough, as when the haemoglobin is reduced, and the ultrastructure weak enough, as in the promeniscocyte, the forces tend first to break, and then to distort, the **C**-shaped rim of the cell into the forms which the meniscocyte successively assumes. The final sickle cell is clearly a form associated with less potential energy than the disk from which it is derived. Diminution of the turgor-producing forces by the oxygenation of the haemoglobin allows the intermolecular arrangements in the surface ultrastructure to re-establish themselves, and the discoidal form is restored, essentially by a progressive curvature of the **C**. Looking in the opposite direction at the change from disk to sphere, the force of surface tension is never strong enough to overcome the rigidity of the surface ultrastructure unless the latter is first modified by the addition of such substances as lecithin, or destroyed by the action of lysins. When this happens, however, the force of surface tension is not only unopposed by the rigidity of the ultrastructure, but is actually aided, by the weak turgor-producing forces associated with oxyhaemoglobin, in bringing about the spherical form.

The existence of three forces instead of two provides an explanation of two hitherto obscure observations in connexion with the shape transformations of the red cell. The first is that 'the sequence of shape changes suggests that the inherent structure of the cell is subject to the action of obscure forces which develop during the haemolytic process, and which disappear when lysis takes place' (Ponder, 1942*a*).

* The total surface area of the sickle cell is probably greater than that of the disk from which it is derived, but it is impossible to measure it with any degree of accuracy. It is certainly much 'straighter', i.e. the mean curvature is less. The red cell in sickle-cell anaemia is more resistant to haemolysis by hypotonic saline than is the normal human red cell. In spite of a prevalent idea that red-cell shape can be inferred from measurements of resistance to hypotonic haemolysis, this is not to be interpreted as evidence that the surface of either the promeniscocyte or the meniscocyte is greater than that of the normal cell. Because of the observation that red cells of different degrees of flatness haemolyse at different critical volumes, attained in different tonicities (Gansslen, 1922; Haden, 1934; Castle & Daland, 1937; Ponder, 1937), the inference that increased resistance means increased flatness can sometimes be made, but only when factors such as the critical volume at which the cells haemolyse, the way in which volume change is related to tonicity change, etc., remain constant. That they do not always do so is shown by the fact that the critical volume in hypotonic NaCl is smaller than that in hypotonic plasma (Ponder, 1937), and the observation that red cells do not always swell as 'perfect osmometers' (Ponder, 1940; Guest & Wing, 1942).

* The essential factor for the development of the expansive forces seems to be the passage of the haemoglobin from the combined to the reduced state, for the sickle cells become disks when not only oxygen, but also CO, is admitted to the preparation. The forces are thus peculiarly associated with reduced haemoglobin.

These are probably the weak turgor-producing forces associated with the haemoglobin in the interior, and they are probably not forces which develop, but rather forces the effect of which is first seen, during the process of weakening of the surface ultrastructure and the subsequent haemolysis. The second observation is that 'ghosts' are unable to undergo disk-sphere transformation. After haemolysis by water the ghost rapidly assumes the biconcave shape and the volume of the cell from which it was derived, but no sphering can be observed between slide and slip, with lecithin, with saponin even in concentration as great as 10%, with bile salts, or with rose bengal. The molecules of the surface membrane, distorted in the stretched prolytic sphere, return to their original positions when the stretching forces disappear at the moment of lysis, after which, the original structure re-established, the surface structure seems to undergo something analogous to 'permanent set' in its biconcave form (Ponder, 1942*b*). The most likely explanation of this inability of the ghost to form a sphere is that the forces associated with the haemoglobin (type III) disappear as a result of the haemolysis, while the surface tension forces (type I) become equal inside and outside the surface ultrastructure. As a result even weak forces (type II) in the ultrastructure of the ghost are able to restore and maintain its discoidal form, and the addition of lecithin, etc., does not result in sphere formation because there are no forces of types I and III remaining.

In the meantime the nature of the turgor-producing forces associated with haemoglobin and its reduction to reduced haemoglobin must remain unanswered. It may turn out that these forces are peculiar to the haemoglobin of the promeniscocyte, just as its unstable surface ultrastructure seems to be.* The shape changes, however, raise in an acute form the question whether the structure of the red cell is as homogeneous as is usually supposed, and whether the materials and structure, both at the surface and in the interior, are the same in the neighbourhood of the biconcavities as they are at the peripheral rim. Leptoscopic measurements have already shown that the red cell envelope is thicker by some 30–40 A. in the region of the biconcavities than it is elsewhere, and that the difference can be made even more pronounced by extraction with organic solvents (Waugh & Schmitt, 1940); the new question now arises whether the haemoglobin is necessarily distributed uniformly throughout the cell, either as a highly viscous solution or as a gel, because we have to account for its absence from the veil-like material of the sickle cell, a material which corresponds to that of the biconcavity of the disk, and for its return to this region when the sickle-cell transformation is reversed. One type of explanation is that the forces developed during the formation of the sickle cell may be such as to bring the surface ultrastructures in the region of the biconcavities into apposition, so that they drive the pigment out from between them into the body of the meniscocyte; on the transformation being reversed, the surfaces would separate, and the haemoglobin would flow back between them into what would be the biconcavity of the reconstituted disk. This explanation retains the idea of the haemoglobin as being in solution, although it makes it necessary to suppose that the enveloping membrane has a highly heterogeneous structure. An alternative type of explanation is that the pigment is not in solution, but is combined with a cytoplasmic matrix or even imbedded in it in a non-uniform manner*, and that it leaves and returns to the region of the biconcavities by a process similar to protoplasmic streaming. Auer (1932) gives a description of some phenomena observed in rabbit and guinea-pig cells which suggest this possibility (his Fig. 2 I, K).

One's choice of these two types of explanation will depend on whether one prefers to think of heterogeneity of structure as a property of the surface ultrastructure or as a property of the red cell interior, and the two types of explanation are not mutually exclusive. The decision would be greatly simplified if there were incontrovertible evidence, one way or the other, as to the physical state of the haemoglobin in the red cell interior, but this, to my mind, is still an open question. The heterogeneity of structure itself, however, seems to be an absolute necessity in the case of the meniscocyte and the promeniscocyte, and the fact that the promeniscocyte is indistinguishable morphologically from the normal human red cell raises the same kind of questions regarding the structure of the latter.

SUMMARY

This paper is concerned with the shape transformations of the promeniscocyte, a cell morphologically indistinguishable from the normal human erythrocyte, into the meniscocyte or sickle cell. This shape change occurs when promeniscocytes are exposed to reduced O_2 tensions, and is completely reversible.

* Both Gough and Teitel-Bernard have suggested that the flattened form of the mammalian red cell is due to the haemoglobin existing as a liquid crystal with expansive forces in the plane of the equatorial axis. That this is not the whole explanation for the discoidal shape is shown by the fact that the watery ghost is discoidal, but it may be that a biconcave discoidal ultrastructure accommodates haemoglobin arranged in a liquid crystal of the same general shape, and giving rise to forces of type III because of a tendency to expand in the equatorial plane.

* A number of instances are known in which the haemoglobin is not uniformly distributed throughout the red cell, at least when it is fixed and stained. The best examples are the erythroblast in the stage of haemoglobinization, the target cell, and the cell of Mediterranean anaemia (Cooley).

Although there must be some essential structural difference between the promeniscocyte and the normal red cell, the transformation throws an indirect light on several problems connected with red cell shape. These are discussed, with the general conclusion that a high degree of heterogeneity must be supposed to exist either in the surface structure or in the interior of the promeniscocyte and the sickle cell, and possibly in the surface or in the interior of the normal erythrocyte as well.

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THE NUTRITION OF THE LARVA OF *AEDES AEGYPTI* L. I

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(*Received* 24 *October* 1944)

Insects, because of their great diversity of form and habit, offer a very wide field for physiological research. Apart from the purely academic aspect it is obvious that there is much more hope of controlling harmful insects or encouraging beneficial ones if their life processes are more thoroughly understood. There is also the possibility that some of these life processes are similar or related to those of the vertebrates including man himself, in which case insects may yield interesting and valuable information of more direct importance. In fact, when more is known about them there appears to be no reason why some should not come into use as laboratory animals. A start in this direction has already been made since the larva of the wax moth *Galleria mellonella* has been used for the estimation of nicotinic acid (Rubinstein & Shekun, 1939).

From a practical point of view the nutrition of mosquito larvae may be of great importance. It is quite possible, for example, that the lack of certain essential growth factors may explain the well-known problem of the absence of larvae from an apparently suitable breeding place. If this is so then the possibility of inhibiting or destroying one or more of these essential growth factors, as a measure of control, suggests itself.

In nature some of the growth factors required by mosquito larvae are synthesized for them by other organisms living in the same medium; but as other non-essential substances are no doubt synthesized at the same time it becomes necessary to perform all experiments under sterile conditions.

A considerable amount of work on the nutrition of the larva of *Aedes aegypti* has already been done. Two very important facts have come to light as a result of this work, first that this larva can make use of solutes (Hinman, 1930), and secondly that it can complete its life cycle from egg to adult in a medium of yeast and liver free from living micro-organisms (Trager, 1935).

Since yeast and liver are apparently adequate for successful larval growth and metamorphosis, the purpose of our investigations has been to replace these with known substances, in other words, to provide the larvae with a completely synthetic diet.

The present paper deals with the technique used by us and certain preliminary investigations on the basic medium.

MATERIAL AND METHODS

Fresh brewer's yeast was obtained from the Castle Breweries, Johannesburg. The compressed yeast was suspended in 0·01 *M* $CaCl_2$, and in this way 0·5 g. of yeast introduced into each tube. Dried brewer's yeast from the same source had been dried on rollers at 208° F. and was used in quantities of 0·2 g. per tube. Thiamin-free baker's yeast was prepared by the method of Kline, Hall & Morgan (1941).

The liver extract used throughout this work was made by Armour Laboratories. It was used in 4% concentration in 0·01 *M* $CaCl_2$ and 10 ml. of the solution placed in each tube. Papaic liver digest was prepared by the method of Shaw (Davis, Davidson, Riding & Shaw, 1943) and dried in the frozen state by Dr E. Gaynor Lewis of this Institute in a lyophile serum apparatus. The powder, which was completely water-soluble, was used in 2% concentration in calcium chloride solution. Armour's liver extract was rendered thiamin-free by sulphite treatment according to Kline *et al.* (1941). It was used immediately after preparation in the same concentration as the original liver extract.

The vitamin mixture comprised the following vitamins: thiamin (100 μg.), riboflavin (100 μg.), pyridoxin (50 μg.), calcium pantothenate (50 μg.), nicotinic acid (100 μg.), biotin (0·025 μg.), *p*-aminobenzoic acid (100 μg.), *i*-inositol (250 μg.); choline chloride (250 μg.) was not normally included in the vitamin mixture, but was added in some experiments (see later). The quantities given are the amounts dissolved in 10 ml. of solution, i.e. per tube or per larva. Solutions were made up immediately before use and exposed to light as little as possible throughout the course of the experiments.

The *A. aegypti* used were originally obtained from Salisbury Island, Durban, Natal, some years ago. Since that time they have been cultured in an insectary at this Institute, rabbits or guinea-pigs being the source of blood for the females and dried yeast or 'comproid'—a composite food used for feeding laboratory animals—for the larvae.

Method of obtaining sterile eggs and larvae

At first we placed Petri dishes lined with damp filter-paper into the cage containing the adults. After eggs had been laid on the damp paper this was cut

up into strips and placed in White's sterilizing fluid (White, 1931). Cultures of larvae free from bacteria were easily obtained in this way, but moulds were a constant source of trouble. We therefore adopted the following technique which has given very good results. Gravid females caught in the insectary are confined, singly, in sterile test-tubes together with 2–3 ml. of sterile distilled water and placed in an incubator at 28°C. The tubes are examined every morning and the females liberated from those tubes found to contain eggs. The water is removed with a sterile pipette and the eggs left stranded. In this way we rarely used eggs older than 24 hr. Tubes with stranded eggs are now half filled with White's solution and left for 15 min. At the end of this time the sterilizing fluid is removed and the sterile eggs washed several times with sterile distilled water. After each washing the water is removed with a sterile pipette. When the eggs are in the final washing of water they are stirred round with a platinum loop so as to dislodge those adhering to the sides of the tube. After the final washing the eggs suspended in water are poured off into a sterile Petri dish and covered. With a sterile pipette the eggs are removed five or more at a time, introduced into tubes containing agglutinating broth and stored in an incubator at 37°C. On about the third day hatching begins, and by that time if any contaminant is present it will readily be seen in the broth. Uncontaminated tubes are examined daily, larvae removed with a pipette and placed singly into each tube containing the medium which is to be tested. In this way larvae of known age are available for experimental purposes. Larvae do not grow in the agglutinating broth but will remain alive in it for several days.

Careful experiments were carried out on larvae 12, 24 and 96 hr. old when transferred to the same medium. It was found that within these limits the age of the larva has no effect on the rate of growth and survival after transference.

Sterilization and dispensing of media

(1) *Solid fraction.* The tubes containing the yeast were autoclaved for 0·5 hr. at 15 lb. pressure.

(2) *Liquid fraction.* In all experiments the liquid fraction, after adjustment to *p*H 5·8, was passed through a Seitz filter using positive pressure. The sterile filtrate was collected in a special apparatus designed to facilitate rapid and reliable dispensing of the liquid into tubes containing the sterile solid fraction. Lack of space makes a full description of our apparatus impossible.

The tubes were tested for sterility by the usual methods, as a rule at the conclusion of each experiment.

Observations of development and methods of expressing them

The stage of development reached by each larva was recorded every morning and evening, the time taken for the change from one stage to the next being calculated to the nearest half-day. In considering the results of our experiments, therefore, it was possible to compare the times taken for each stage (referred to subsequently as 'growth rates') and also the number of larvae reaching that stage (referred to as the 'survival rate'). In doing so we have departed radically from the procedure of Trager (1937) who combined growth and survival rates (only as far as the fourth larval instar) in an arbitrary factor which on occasion served to mask the underlying significance of his findings. Our results have been subjected to statistical treatment, and the validity of our conclusions checked statistically as far as possible. It is realized that the growth rates recorded here are subject to a maximum error of +0·5 day, and that allowance could be made for this error by subtracting 0·25 from the overall times noted for the change from first instar larva to adult. However, such a step would not affect the standard deviations nor the significance of differences between groups. It has consequently been decided to record the times at which the changes were observed and to regard these as representing the times taken for the changes from one stage to the next. A typical example of the manner in which our observations were made is given in Table 1 below. In subsequent tables the results have been condensed, but all the data are available in the same detail. By keeping the larvae under such close and continuous observation, we have been able to draw inferences which would not otherwise have been possible.

EXPERIMENTAL

Attempts to improve basal medium

Our initial experiments were concerned with the investigation of various basal media. A suspension of fresh brewer's yeast in liver extract solution formed the starting-point. Efforts were made to discover whether further additions to this medium brought about any improvement in the growth rate. A mixture of vitamins (listed previously) was first added to the medium, but no such effect was observed. Similarly, replacement of liver extract by papaic liver digest also brought about no improvement, even on supplementing with the vitamin mixture (Table 1).

If, in place of fresh brewer's yeast, roller-dried brewer's yeast was employed in conjunction with liver extract, a slight, but statistically significant difference in the growth rate was observed, particularly in the length of time required for pupation (Table 1). Addition of the vitamin mixture here, too, brought about no improvement.

Effect of omission of liver extract from medium

The possibility was explored of omitting liver extract from our media or of replacing it by some simpler mixture of substances. A medium consisting of autoclaved fresh brewer's yeast suspended in

Table 1. *Rate of growth and survival of mosquito larvae: the effect of various supplements added to autoclaved fresh and dried brewer's yeasts*

Basal medium. Autoclaved fresh or dried brewer's yeast (0·5 g.) and 10 ml. 0·01 *M* $CaCl_2$ solution.
No. Number in stage of development indicated.
Mean, S.D. Mean and standard deviation of time (in days) taken to change from the previous stage.

Additions to basal medium	No. of experiments	Larval instars							Pupae		Adults	
		I	II		III		IV					
		No.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.
I. Fresh brewer's yeast medium:												
None	3	60	58	1·8 ± 0·37	56	2·8 ± 1·37	52	5·6 ± 2·66	48	12·1 ± 4·85	43	2·4 ± 0·57
Liver extract	1	20	19	2·0 ± 0·00	19	1·0 ± 0·51	18	0·8 ± 0·30	18	2·5 ± 0·20	17	2·2 ± 0·30
Liver extract + vitamins	1	20	20	1·9 ± 0·62	20	0·9 ± 0·20	20	1·0 ± 0·30	19	2·7 ± 0·47	19	2·2 ± 0·25
Papaic liver digest	1	30	29	2·1 ± 0·55	29	1·1 ± 0·31	29	1·3 ± 0·31	29	2·8 ± 0·79	29	2·1 ± 0·22
Papaic liver digest + vitamins	1	20	18	1·9 ± 0·72	18	1·2 ± 0·37	18	1·25 ± 0·30	18	3·3 ± 0·80	18	2·25 ± 0·25
II. Dried brewer's yeast:												
None	3	60	55	1·7 ± 0·43	54	1·0 ± 0·26	53	1·7 ± 0·54	52	4·4 ± 1·38	50	2·2 ± 0·49
Liver extract	4	110	98	2·1 ± 0·63	95	1·4 ± 0·54	93	1·4 ± 0·75	92	3·6 ± 1·36	89	2·5 ± 0·59
Liver extract + vitamins	2	40	39	2·2 ± 0·33	39	1·4 ± 0·66	34	1·3 ± 0·46	32	4·0 ± 0·97	30	2·7 ± 0·44

Table 2. *Rate of growth and survival of mosquito larvae on media containing various forms of brewer's yeast*

Medium		Length of storage at room temperature after autoclaving	Length of autoclaving at 15 lb. pressure	No. of experiments	No. of first instar larvae	No. of adults emerging	Time (in days) taken to reach adult stage	
Liquid fraction	Solid fraction (Form of brewer's yeast employed)						Range	Mean S.D.
0·01 *M* $CaCl_2$	Fresh	None	15 min.	3	60	43	11 –49	20·9 ± 5·87
	Roller-dried	None	15 min.	3	60	50	7·5–15·5	10·8 ± 1·65
	Roller-dried	6 months–2 years	15 min.	2	40	24	8 –47·5	16·3 ± 7·28
	Autoclaved fresh	20 days	15 min.	1	20	2	22 –26	24·0
Liver extract + vitamins	Fresh	None	15 min.	1	20	19	7·5–12·5	8·7 ± 1·19
	Autoclaved fresh	17 days	15 min.	1	20	10	9 –14·5	10·5 ± 1·70
	Fresh	None	5 hr.	1	20	15	9 –18·5	12·1 ± 2·53
	Fresh	None	10 hr.	1	20	5	8·5–16·5	12·8 ± 2·05

0·01 *M* $CaCl_2$ solution was capable of supporting growth of larvae, the proportion reaching maturity being 65–95%. The *rate* of growth was, however, greatly retarded (Table 1). Roller-dried brewer's yeast with calcium chloride gave somewhat anomalous results. It was found, for instance, that when used shortly after being dried, the yeast produced a better rate of growth than fresh yeast had done. When the dried yeast was stored for 6 months to 2 years before use, a longer time was needed for the adult stage to be reached (Table 2). It appeared that some unstable factor was present in yeast which, while it decomposed slowly in the dry material, disappeared rapidly when in contact with liquid. A simple experiment lent support to this view. When fresh brewer's yeast was autoclaved and kept at room temperature for 20 days before use, an extremely poor rate of growth and survival was observed (Table 2). Further experiments, not included in Table 2, appeared to indicate that the conditions of drying of brewer's yeast affected its growth-promoting properties.

Effect of prolonged storage and autoclaving of yeast

In order to ascertain whether the unstable factor was one of the B group of vitamins, the fresh brewer's yeast was once more combined with filtered liver extract and the previously mentioned vitamin mixture. Keeping the autoclaved yeast for 17 days before use produced a similar, though not nearly so marked, effect as it had done in the absence of liver extract. In the same way, autoclaving the yeast for 5 hr. before use had only a slight adverse influence on the growth and survival rates, the effect on survival rate being more pronounced when autoclaving was carried on for 10 hr. (Table 2).

Experiments with thiamin-free yeast and thiamin-free liver extract

Similar observations were made in a series of experiments carried out in another connexion, but which may be referred to here, since they serve to illustrate the same point. In a medium comprising thiamin-free baker's yeast and thiamin-free liver extract no growth occurred beyond the second larval instar. In tubes containing the freshly prepared medium, addition of thiamin (25 or 50 μg. per tube) restored the growth and survival rates almost to normal. When thiamin-free yeast was used which had been prepared at least 6 months before, few larvae reached maturity, even on addition of 100 μg. thiamin per tube. Autoclaving of the dry freshly prepared yeast for 3 hr. had the same effect as prolonged storage (Table 3). The influence of both treatments was most marked at the pupal stage, at which the medium appeared to become quite inadequate.

Replacement of liver extract by mixtures of vitamins

To bring out this effect more clearly, the liver extract was replaced by the vitamin mixture previously employed. Fresh yeast, newly dried and old dried yeasts were tried in turn (Table 4). It was found that in combination with fresh yeast, liver extract could be completely replaced by the vitamin mixture. With dried yeast the addition of vitamins, while it served to replace liver extract, did not succeed in increasing the growth rate to the same level as observed with the fresh yeast-vitamin combination. The addition of choline chloride had no effect in the fresh yeast medium but brought about a significant improvement in the dried yeast medium. This is difficult to account for at present.

Table 3. *Experiments with thiamin-free yeast and thiamin-free liver as the basal medium*

Length of storage of thiamin-free yeast	Length of autoclaving of thiamin-free yeast at 15 lb. pressure	Quantity of thiamin added per tube μg.	Larval instars			Pupae		Adults	
			I	II–IV					
			No.	No.	Time taken from first to fourth larval instars Mean S.D.	No.	Mean S.D.	No.	Mean S.D.
None	15 min.	0	49	0	—	—	—	—	—
None	15 min.	25–50	44*	44	6·1 ± 0·56	44	4·2 ± 0·63	44	2·0 ± 0·39
6 months	15 min.	100	50	44	6·9 ± 0·92	8	4·4 ± 0·77	6	2·3 ± 0·47
18 months	15 min.	33	50	36	7·9 ± 1·23	1	7·0	1	3·0
None	3 hr.	50–100	90*	82	6·0 ± 0·75	3	4·0	2	2·3

* Number given represents total of two experiments.

Replacement of yeast by other substances

Unsuccessful attempts were made to replace yeast by other materials used in conjunction with a solution containing liver extract and vitamins (including choline chloride). Among the substances employed were: charcoal, peptone, casein, casein hydrolysate and mixtures containing some or all of the following: glucose, tryptophane, tyrosine, cystine, ascorbic acid, glutathione, adenine, guanine and uracil. In the

media containing casein and peptone a small proportion of larvae reached the second instar; in peptone one larva even reached the third instar after 35 days. In all the other media no growth occurred; in many the first-instar larvae remained alive for periods up to 21 days but did not moult.

2 and 3 days, the mean being $2{\cdot}4 \pm 0{\cdot}46$ days. Of those pupae, 97% emerged as adults, indicating that as a rule emergence also is unaffected by the medium.

One final observation must be recorded here. In the insectary it is most unusual to find a weak adult

Table 4. *The effect of replacement of liver extract by mixtures of vitamins*

Medium		No. of experiments	No. of first instar larvae	No. of adults	Time (in days) taken to reach adult stage	
Solid fraction	Liquid fraction				Range	Mean S.D.
Fresh brewer's yeast	Liver extract	1	20	17	8 – 9	$8{\cdot}4 \pm 0{\cdot}28$
Fresh brewer's yeast	Vitamin mixture	2	38	32	7·5–17·5	$9{\cdot}0 \pm 1{\cdot}53$
Fresh brewer's yeast	Vitamin mixture + choline chloride	2	40	30	7·5–10·5	$8{\cdot}6 \pm 0{\cdot}57$
Dried brewer's yeast	Liver extract	4	110	89	7 –19·5	$10{\cdot}5 \pm 1{\cdot}28$
Dried brewer's yeast	Vitamin mixture	3	59	50	8 –19	$11{\cdot}0 \pm 1{\cdot}83$
Dried brewer's yeast	Vitamin mixture + choline chloride	2	40	37	7·5–10	$8{\cdot}5 \pm 0{\cdot}96$

DISCUSSION

From the above experiments it would appear that brewer's yeast alone, when autoclaved fresh and suspended in calcium chloride, can provide all the nutritive factors required by the larva of *A. aegypti*. Addition of B vitamins in solution, by making some of these growth factors more readily available, speeds up growth to a rate equal to that observed in our best media.

However, it is clear from our results that yeast plays a more fundamental part than merely to supply protein, B vitamins and mineral salts. The presence has been demonstrated of a heat-labile factor, which is destroyed on autoclaving the yeast or keeping it even in the dry state. A heat-stable factor or factors must also be present; these are not supplied by other possible sources such as casein and peptone. Both factors are essential for adequate growth and metamorphosis. The heat-stable factor is absolutely essential, but growth appears to take place, at any rate to some extent, when the heat-labile factor has been largely or completely destroyed.

Subbarow & Trager (1940) found that they could partially replace the liver fraction in a yeast-liver medium with pyridoxin, pantothenic acid, riboflavin and glutathione. We have not found added glutathione necessary and have been able to replace the liver fraction entirely with a mixture of known vitamins.

In the wide variety of media used, both in the work reported here and in other experiments, it has been noted that the time taken for the change from pupa to adult is independent of the diet. The results of sixty experiments have been correlated, involving over 1000 pupae. The interval between pupation and emergence has been found to range between

mosquito, that is, one which on emerging cannot rise from the water. In our experiments, however, even on the most complete media, a large proportion of the adults were weak irrespective of sex. There is thus some further factor, whose nature we have not yet investigated, which is responsible for the development of vigorous adult mosquitoes.

SUMMARY

1. A technique for investigating the nutritional requirements of the larva of *Aedes aegypti*, under sterile conditions, is described. A method for more accurate recording and interpretation of results is presented.

2. First-stage larvae may be kept in agglutinating broth for periods up to 96 hr. without affecting their subsequent growth in suitable media.

3. A suspension of autoclaved brewer's yeast in calcium chloride solution supports growth and metamorphosis of the larvae. The addition of filtered liver extract greatly improves the growth and survival rates. Further addition of a mixture of vitamins of the B group brings about no corresponding further improvement; neither does replacement of the liver extract by papaic liver digest with or without the vitamin mixture.

4. Storage of dried brewer's yeast for a long period, or of autoclaved fresh brewer's yeast for a shorter period, has a marked adverse effect on the growth rate when these yeasts are combined with liver extract or vitamin mixture. A similar effect is brought about by prolonged autoclaving of the yeasts.

5. In all these media liver extract may be replaced by the mixture of B vitamins. It was not found possible, however, to replace the solid yeast fraction of the medium by any combination of substances so far investigated.

6. The time taken for the change from pupa to adult is independent of the medium in which the larva was grown.

7. There is a factor which causes the emergence of vigorous adults in nature or in contaminated media. This factor has been found to be largely absent from our best media such as brewer's yeast and liver.

We are indebted to Misses L. O'C. Black and S. Sussman for technical assistance and to the Castle Breweries, Johannesburg, for supplies of brewer's yeast. We wish to thank the Commanding Officer, Technical Staff, Medical Laboratory Service, S.A.M.C., who enabled one of us (M.L.) to partiticiptate in this work. We are grateful to Messrs H. D. Barnes and C. W. Kops for statistical advice.

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THE NUTRITION OF THE LARVA OF *AEDES AEGYPTI* L.

II. ESSENTIAL WATER-SOLUBLE FACTORS FROM YEAST

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(*Received* 27 *November* 1944)

The study of the nutritional requirements of mosquito larvae has made comparatively little progress since Trager (1935) reported that they could be brought to maturity in a sterile medium consisting only of autoclaved yeast and liver extract. Subsequently a number of random observations were reported, dealing with the factors necessary for growth and metamorphosis of mosquito larvae. No concerted effort, however, has been made to replace the yeast-liver extract medium of Trager by a mixture of known components. This is the immediate object of our investigations, and the results presented below serve to bring it yet another stage nearer realization.

In the first paper of this series, De Meillon, Golberg & Lavoipierre (1945) described an improved technique for studying the nutrition of mosquito larvae and laid emphasis on the need for frequent observations on the stage of development reached by each larva. Preliminary research revealed that further enrichment of the yeast and liver extract medium failed to improve the rate of growth. Liver extract could be replaced completely by a mixture of synthetic vitamins of the B group and even, to some extent, by 0·01 *M* $CaCl_2$. It was not found possible, however, to replace the solid yeast fraction of the medium.

The growth-promoting properties of autoclaved yeast were found to be adversely affected by prolonged autoclaving or storage, even if thereafter the yeast was combined with liver extract or the mixture of vitamins previously employed. The present investigation deals with the water-soluble factors in yeast which are essential for growth and survival of mosquito larvae.

EXPERIMENTAL

The materials and methods employed in this work have been described, as fully as restrictions on space have permitted us to do so, in Part I (De Meillon *et al.* 1945). There follows for the most part, only such information as did not appear in our previous communication.

The materials used in various liquid media are given below, the concentration per litre of liquid medium being represented by the figure in brackets after each substance.

Liquid fraction of basal medium (*vitamin mixture*). Thiamin (10 mg.), riboflavin (10 mg.), pyridoxin (5 mg.), calcium pantothenate (5 mg.), nicotinic acid (10 mg.), biotin (10 mg.), *p*-aminobenzoic acid (25 mg.), *i*-inositol (25 mg.), choline chloride (25 mg.) and Tatum's salt mixture (Tatum, 1939) (1·5 g.).

The biotin concentrate used in our earlier work was prepared according to the directions of Snell & Wright (1941). Later the pure free acid was made available to us by Eli Lilly and Co.

Other components of the medium (*liquid fraction*). A concentrate of the ammonium salt of folic acid of potency 5000 was obtained from Prof. R. J. Williams and used in a concentration of 20 mg./l. The 'Norite eluate factor' of Peterson (Hutchings, Bohonos & Peterson, 1941) was supplied by Lederle Laboratories, and was used at a level of 12 mg./l. A concentrate of vitamin B_c was prepared from liver according to the directions of O'Dell & Hogan (1943), the preliminary aqueous extract being dried in the frozen state by Dr E. Gaynor Lewis of this Institute in a lyophile serum apparatus. The concentrate was used at a level of 250 mg./l.

Vitamin K was supplied in water-soluble form, as the tetrasodium salt of 2-methyl-1:4-naphthohydroquinone diphosphate (25 mg./l.) provided by Roche Products, Ltd.

Other pure substances employed were: glucose (20 g.), ascorbic acid (50 mg.), glutathione (50 mg.), yeast nucleic acid (250 mg.), adenine (10 mg.), guanine (10 mg.), uracil (10 mg.), thymine (200–1000 mg.), cystine (10 mg.), tryptophane (10 mg.) and tyrosine (10 mg.).

Xanthopterin was synthesized by two procedures, both of them modifications of the method of Purrmann (1940). The first was that of Totter (1944) and the other our own. The two samples possessed identical absorption curves and were used either in a concentration of 200 mg./l. or in supersaturated solution which deposited solid material on standing.

Avidin was prepared according to the directions of Eakin, Snell & Williams (1941) and used immediately after dialysis, without acetone precipitation. The avidin solution resulting from 0·6 l. fresh egg white was dissolved in 250 ml. liquid medium.

Yeast autolysates, extracts and residues. The autolysate and residue were prepared from fresh brewer's yeast by heating 1 kg. with 1 l. 0·01 *N* HCl for 24 hr.

at 70° C. The residue was separated by centrifugation and washed twice with water. The autolysate was passed through a clarifying pad and sterilized by filtration. It was incorporated in the liquid medium in the proportion of 100 ml./l.

Dried brewer's yeast was autolysed by heating 275 g. with 1 l. water at 50° C. for 24 hr. and the autolysate also used in a concentration of 100 ml./l.

The cold-water extract of fresh brewer's yeast was prepared by stirring 100 g. yeast violently with 200 ml. of water at room temperature for 4 hr. The extract was separated by centrifugation and 100 ml./l. placed in the medium.

Water-insoluble yeast residue was prepared by boiling fresh brewer's yeast with 2 vol. of water and a few drops of caprylic alcohol for 10 min. After centrifugation the process was repeated 3 times using 1 l. of water for each litre of original yeast. The final centrifuged residue from these extractions was extracted continuously for 48 hr. with boiling water in a Soxhlet apparatus. The residue after centrifugation was dispensed into tubes (0·2 g. suspended in 1 ml. H_2O in each tube) and autoclaved immediately.

Liver preparations. Armour Laboratories liver extract (40 g./l.) was used throughout this work. Papaic liver digest (20 g./l.) was prepared as described in our previous paper.

Methods. The same scrupulous precautions to ensure sterility which were observed in our previous work were even more necessary in these experiments. The general methods of sterilizing liquid media by filtration and solids by autoclaving remained unchanged. Observations were made and results recorded as described before (De Meillon *et al.* 1945).

The microbiological assay method of Landy & Dicken (1942) was employed for estimating biotin in water-extracted yeast.

RESULTS

Effects of autolysis and extraction of fresh brewer's yeast. The effects of autolysis and aqueous extraction on the growth-promoting properties of fresh brewer's yeast were first investigated. It was found (Table 1*b*) that growth was considerably retarded on media containing the wet, that is undried, autolysis residue in place of the yeast itself (Table 1*a*). Furthermore, no improvement was brought about by the addition of the autolysate to a liquid fraction composed of liver extract and vitamin mixture (Table 1*c*). This seemed to indicate that the autolysate contributed nothing which was not already present in the residue or in the liquid medium.

That the yeast residue was essential for growth was demonstrated in numerous experiments not recorded in the tables. In the absence of yeast residue the combination of liver extract and vitamin mixture was unable to promote growth, even to the second instar. The addition of yeast autolysate, cold aqueous yeast extract or powdered charcoal proved of no avail.

The dried autolysis residue prepared from fresh brewer's yeast possessed even poorer growth-promoting properties than did the wet, freshly prepared, material (Table 1 *d*, *e*). The addition of cold aqueous yeast extract did not serve to improve the rate of growth (Table 1*f*). On the other hand, combination of dried autolysis residue with yeast residue from cold aqueous extraction did much to restore the rate of growth (Table 1*g*).

These results should be interpreted in conjunction with the influence of the media on the proportion of larvae surviving. It appeared probable that autolysis of fresh brewer's yeast had served to liberate or produce some water-soluble agent exhibiting a marked toxicity towards mosquito larvae. This toxicity bore little relation to the rate of growth,

Table 1. *Growth of mosquito larvae on media containing fresh brewer's yeast autolysates and residues* (20 larvae in each experiment)

No. of exp.	Medium		No. of adults	Time (in days) taken to reach adult stage	
	Solid fraction	Liquid fraction		Range	Mean S.D.
a	Fresh brewer's yeast	Liver extract + vitamins	19	7·5–12·5	8·7 ± 1·19
b	Autolysis residue (wet)	Liver extract + vitamins	12	8 –16	13·4 ± 1·28
c	Autolysis residue (wet)	Autolysate + liver extract + vitamins	11	12 –17·5	13·5 ± 1·45
d	Autolysis residue (dried)	Liver extract + vitamins	19	12·5–19	15·1 ± 1·43
e	Autolysis residue (dried)	Autolysate + liver extract + vitamins	14	10·5–27·5	15·4 ± 3·87
f	Autolysis residue (dried)	Autolysate + liver extract + vitamins + cold aqueous yeast extract	7	13·5–20·5	17·3 ± 2·45
g	Autolysis residue (dried) + cold aqueous extraction residue	Autolysate + liver extract + vitamins	8	9·5–19·5	12·0 ± 2·93
h	Autolysis residue (extracted)	Liver extract + vitamins	16	14·5–23	18·8 ± 2·72

as was shown by the better survival rate when dried autolysis residue replaced the fresh form. Inclusion in the liquid fraction of the medium of autolysate with or without cold aqueous yeast extract increased the mortality of the larvae, even when the growth rate was greatly improved by addition of cold aqueous extraction residue.

Prolonged extraction of the autolysis residue with boiling water reduced its growth-promoting properties still further, but the proportion of larvae surviving was high (Table 1*h*).

The above experiments demonstrated the probable existence in fresh brewer's yeast of two factors, one essential for optimum growth, the other responsible for toxic effects leading to increased larval mortality. The presence of the growth-promoting factor was established in experiments previously reported (De Meillon *et al.* 1945). The conclusion may be drawn from Table 1 that drying of fresh brewer's yeast or of the autolysis residue prepared from it serves to counteract the toxic effects, which are not destroyed during ordinary autoclaving.

medium investigated comprised insoluble yeast residue and vitamin mixture. Some results are given in Table 3.

This medium permitted growth to the fourth larval instar but not beyond it. The addition of glucose, other vitamins, glutathione, yeast nucleic acid, amino acids and some purine and pyrimidine bases, either singly or in various combinations, did not serve to bring about pupation. Only liver extract or papaic liver digest made it possible for pupation to occur.

Growth on papaic liver digest was considerably better than on liver extract. In fact the combination of papaic digest and vitamins was found to be capable of supporting fair growth to the adult stage. To the best of our knowledge, this is the first recorded instance of the culture of mosquito larvae to the adult stage under sterile conditions in a completely liquid medium.

A new medium was next combined with insoluble yeast residue. It consisted of the vitamin mixture, glucose, glutathione and yeast nucleic acid. The effect

Table 2. *Growth of mosquito larvae on media containing dried brewer's yeast autolysates and residues* (50 larvae in each experiment)

Medium		No. of adults	Time (in days) taken to reach adult stage	
Solid fraction	Liquid fraction		Range	Mean S.D.
Dried brewer's yeast	Liver extract + vitamins	47	7 –15·5	9·3 ± 1·13
Autolysis residue (wet)	Liver extract + vitamins	44	9 –15	10·2 ± 1·04
Autolysis residue (wet)	Liver extract + thiamin	39	9 –13	9·9 ± 1·07
Autolysis residue (wet)	Autolysate + liver extract + vitamins	40	8·5–16	10·1 ± 0·96
Autolysis residue (wet)	Autolysate + liver extract + thiamin	42	8 –14·5	9·9 ± 1·30
Organism 8*x*	Autolysate + liver extract + thiamin	29	9·5–13	10·5 ± 1·27

Dried brewer's yeast autolysate and residue. The possibility was therefore investigated of inactivating the toxic factor while attempting to preserve as much as possible of the growth factor. To this end an autolysate was prepared from dried brewer's yeast under milder conditions than before. From the results recorded in Table 2 it will be clear that under these conditions the rates both of growth and survival were hardly affected by the autolysis of the yeast. The autolysis residue, whether combined with autolysate, liver extract and vitamins or merely with liver extract and thiamin, gave almost optimum growth.

In Table 2 is included one experiment which showed that a light suspension of sterile bacteria could replace the autolysis residue. The question was not pursued further, however.

Supplementary factors necessary for growth on insoluble yeast residue. From this point we turned to the problem of the nutritive factors which it is necessary to add to the water-insoluble residue from yeast in order to attain optimum growth. The first was studied of omitting the components of the medium one at a time. The results, given in Table 4, were strikingly clear-cut. Once again the absence from the medium of some factor necessary for pupation was clearly shown. Five vitamins—thiamin, riboflavin, pyridoxin, pantothenic acid and nicotinic acid—were absolutely essential for growth, even to the fourth stage. *p*-Aminobenzoic acid, biotin, *i*-inositol, choline and glutathione did not appear to affect growth.

Factors responsible for bringing about pupation. The characteristic of Tables 3 and 4 most worthy of note is that in the presence of the water-insoluble fraction of yeast *pupation did not occur* unless some form of liver was added to the medium. Although the media employed were in many cases capable of supporting good, though somewhat delayed, growth to the fourth stage, the active, fully grown larvae died in the fourth instar, usually after surviving for periods up to 6 weeks. The regularity with which failure to pupate was observed under these conditions is indicated by the fact that in forty-eight experiments

using insoluble yeast residue and involving a total of over 1000 larvae, of which about two-thirds reached the fourth instar, less than 1 % were transformed into pupae.

The basal medium in further investigations consisted of insoluble yeast residue, glucose, salt mixture, yeast nucleic acid, thiamin, riboflavin, pyridoxin, nicotinic acid and pantothenic acid. The effect was studied of adding various other compounds to this medium. These included vitamins, amino acids and purine and pyrimidine bases. Some of the results obtained are given in Table 5.

Only three preparations were found capable of bringing about pupation. These were concentrates of folic acid, of vitamin B_c and of the 'Norite eluate factor'. The three factors induced pupation with such remarkable regularity as to emphasize the close relationship known to exist between them and to indicate their possible identity.

It is known that xanthopterin may be present in concentrates of folic acid (Mitchell, 1943). Wright & Welch (1943) and Mitchell (1944) have provided evidence of a structural similarity between the molecules of xanthopterin and folic acid. On testing

Table 3. *Effect of various additions to a basal medium of insoluble yeast residue and vitamins*

No. = Number in stage of development indicated.
Mean, S.D. = Mean and standard deviation of time (in days) taken to change from the previous stage.

Additions to liquid fraction of basal medium	Larval instars							Pupae			Adults	
	I	II		III		IV						
	No.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	Nc	Mean S.D.		No.	Mean S.D.
None	40	37	2·8 ± 1·24	36	2·9 ± 1·12	16	9·6 ± 6·18	0	—		—	—
Glucose	20	15	1·9 ± 0·76	15	3·0 ± 0·28	13	7·3 ± 2·30	0	—		—	—
Glucose + ascorbic acid	20	18	2·0 ± 0·63	17	2·1 ± 1·10	14	6·3 ± 3·16	0	—		—	—
Glucose + glutathione	20	20	1·8 ± 0·62	19	2·2 ± 0·63	14	4·3 ± 0·97	0	—		—	—
Glucose + glutathione + ascorbic acid	20	18	1·5 ± 0·96	18	2·0 ± 0·30	18	5·3 ± 5·19	1	3·0	—	—	—
Glucose + glutathione + vitamin K	20	15	1·5 ± 0·10	14	1·3 ± 0·36	14	2·6 ± 0·73	0	—		—	—
Glucose + glutathione + yeast nucleic acid	20	20	2·3 ± 1·04	20	1·9 ± 0·44	20	6·0 ± 4·38	0	—		—	—
Glucose + glutathione + adenine + guanine + uracil + cystine + tryptophane + tyrosine	20	18	1·7 ± 0·37	18	1·7 ± 0·41	16	4·5 ± 0·44	0	—		—	—
Liver extract	20	19	3·8 ± 1·19	19	3·7 ± 2·09	19	3·8 ± 1·25	17	6·2	± 1·70	16	2·15 ± 0·30
Papaic liver digest	20	17	1·7 ± 0·39	17	1·1 ± 0·35	17	3·4 ± 0·32	17	3·75	± 1·50	17	2·3 ± 0·30
Medium consisting of papaic liver digest and vitamin mixture only	70	61	4·5 ± 1·62	57	4·5 ± 3·30	48	4·9 ± 2·46	20	9·5	± 1·93	16	2·25 ± 0·50

Table 4. *Effect of withholding single components of the basal medium to which glucose, glutathione and yeast nucleic acid had been added* (20 larvae in each experiment)

No. = number in stage of development indicated.
Mean, S.D. = Mean and standard deviation of time (in days) taken to change from the previous stage.

Component of medium withheld	Larval instars						Pupae		
	II		III		IV				
	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	
None	17	2·3 ± 1·04	17	1·9 ± 0·44	17	6·0 ± 4·38	—	—	
Thiamin	8	2·9 ± 0·69	0	—	—	—	—	—	
Riboflavin	5	2·6 ± 0·58	3	3·3 ± 1·25	0	—	—	—	
Pyridoxin	19	2·0 ± 1·70	11	6·7 ± 2·73	0	—	—	—	
Pantothenic acid	13	4·5 ± 3·16	0	—	—	—	—	—	
Nicotinic acid	17	2·6 ± 0·60	12	5·5 ± 2·51	0	—	—	—	
p-Aminobenzoic acid	20	2·4 ± 0·47	20	2·0 ± 0·71	20	5·6 ± 2·62	1	10·5	—
Biotin	18	2·0 ± 0·65	18	1·9 ± 0·37	17	4·2 ± 1·41	—	—	
i-Inositol	16	1·9 ± 0·38	16	1·5 ± 0·40	14	4·8 ± 2·32	2	8·0	—
Choline	19	2·2 ± 0·57	16	2·6 ± 0·51	15	5·7 ± 2·13	—	—	
Glutathione	15	1·8 ± 0·78	12	2·1 ± 0·35	10	3·6 ± 1·77	—	—	

samples of pure xanthopterin, synthesized as previously described, both failed to bring about pupation or any of the other changes characteristic of folic acid (see below).

There are indications in the literature that thymine shares some of the growth-promoting properties of folic acid, though to a lesser degree (Mitchell & Williams, 1944; Stokes, 1944). The pyrimidine proved to be inactive under the conditions of our experiments (Table 5).

Requirements of other factors. Adding or withholding biotin had no significant effect in all experiments employing insoluble yeast residue. Nevertheless, it appeared likely that biotin was essential for growth of mosquito larvae. In the first place the yeast residue was found by microbiological assay to contain 0·6 μg./g. of bound biotin, which probably became available to the larva on ingestion of the yeast. Secondly, the inclusion of avidin in the medium produced a marked inhibiting effect (Table 5) probably connected with its inhibition of the action of the bound biotin. Definite proof of the correctness or otherwise of these conclusions must await the development of a completely synthetic medium for the larvae.

The need for choline and *i*-inositol remains to be established. While they did not occur in the free state in the water-insoluble yeast, they were present as constituents of the yeast lipoids. Glutathione could not be detected in the yeast and the need for this substance cannot be regarded as established. This contrasts with the finding of Trager (1942).

Without yeast nucleic acid growth was seriously retarded, as was demonstrated by using a complete medium containing folic acid (Table 5). The same medium with nucleic acid and without insoluble yeast supported no growth at all.

Effect of transferring larvae to and from folic acid-free media. In order to gain an insight into the mode of action of folic acid, transference experiments were carried out in which larvae were transferred from a folic acid-free medium to one containing folic acid and vice versa. The groups of larvae were transferred immediately after changing to the second, third and fourth instars respectively and a fourth group kept as controls.

Second- and third-instar larvae transferred to a folic acid medium reached the adult stage in good time. Only a small proportion of fourth-stage larvae were able to pupate and emerge as adults. Those that succeeded in doing so, took a significantly longer time in changing to pupae. Transference of larvae 11 days after changing to the fourth instar in a folic acid-free medium resulted in no visible growth whatever.

The results of the reverse procedure—transfer-

Table 5. *Effect of various additions to the basal medium on the growth of mosquito larvae*

Basal medium. Solid fraction: insoluble yeast residue. Liquid fraction: glucose, salt mixture, yeast nucleic acid, thiamin, riboflavin, pyridoxin, nicotinic acid, calcium pantothenate.
No. = number in stage of development indicated.
Mean, S.D. = mean and standard deviation of time (in days) taken to change from the previous stage.

Additions to basal medium	Larval instars							Pupae		Adults	
	I	II		III		IV					
	No.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.
None	60*	53	2·5±0·96	53	2·8±0·84	38	6·8±2·23	0	—	—	—
Biotin	20	20	2·4±0·69	18	2·1±0·63	12	5·0±2·67	0	—	—	—
Choline chloride	20	20	2·1±0·22	20	2·1±0·67	16	6·1±4·08	0	—	—	—
Glutathione	32†	32	2·2±0·56	32	2·5±1·20	25	6·7±2·50	2	6·0	0	—
i-Inositol	20	15	2·4±0·89	15	2·0±0·59	9	6·4±3·56	0	—	—	—
p-Aminobenzoic acid	20	16	2·3±0·60	16	2·2±0·68	8	5·7±1·30	0	—	—	—
All the above compounds	20	17	2·3±1·04	17	1·9±0·44	17	6·0±4·38	0	—	—	—
Avidin	20	16	3·6±1·97	10	4·5±2·12	2	8·5	0	—	—	—
Thymine	41†	41	1·9±0·22	39	2·1±0·61	16	5·5±1·59	0	—	—	—
Xanthopterin	38†	35	2·3±0·45	35	2·0±0·86	20	5·3±1·49	0	—	—	—
Vitamin B_c	20	19	2·5±0·53	18	1·4±0·45	17	1·6±0·93	15	3·4±0·55	14	2·1±0·82
Norite eluate factor	17	16	2·8±0·75	15	1·8±0·55	15	1·7±0·57	15	2·8±0·73	14	2·1±0·25
Folic acid	59*	59	1·7±0·39	58	1·5±0·37	58	1·5±0·32	55	3·1±0·48	54	2·3±0·37
Folic acid; no yeast nucleic acid	20	5	3·5±2·00	5	2·7±0·87	5	5·1±3·09	2	5·3±1·25	2	2·0
Folic acid; no insoluble yeast residue	20	0	—	—	—	—	—	—	—		
Medium consisting of autoclaved fresh brewer's yeast + liver extract	60*	59	1·8±0·30	59	1·1±0·48	58	1·0±0·30	57	2·5±0·35	55	2·2±0·39

* Average of three experiments. † Average of two experiments.

ence from a folic medium to one free of the vitamin —were in full accordance with the above findings. Larvae transferred in the second stage grew well to the fourth instar; but few succeeded in pupating and then only after a long time. Third-stage larvae fared better, and the great majority of fourth-stage larvae completed their growth to adulthood in good time. In most of these experiments, however, the rate of growth was markedly delayed as compared with that of larvae constantly in media containing folic acid.

Other effects of factors bringing about pupation

The concentrates which were found to be capable of inducing pupation brought about several other effects. Three of the most striking characteristics of larvae grown in folic acid-free media were: the entire absence of bodily pigmentation, with the exception of the head and siphon which were normally pigmented; the small size of the fourth-instar larvae; and the inability of many, usually of most, of the fourth-stage larvae to disengage completely their third-instar pelts. Often such larvae died with the pelts still attached. In addition, a less striking but none the less real effect was a delayed rate of growth, particularly for the change from third to fourth instars (seen most clearly in Table 6).

The addition of concentrates of folic acid, vitamin B_c or of 'Norite eluate factor' led to fourth-stage larvae which appeared to be normal in every respect.

The presence of xanthopterin in solution in the medium, although it failed to bring about pupation, led to a bright red pigmentation of the cardiac caecae. Where particles of xanthopterin separated on the surface of the yeast, they were eaten up completely, the granules appearing in the intestinal tract but not in the cardiac caecae.

In one of the media composed of papaic liver digest and vitamin mixture (see bottom of Table 3) the cardiac caecae stood out dark in contrast to the head and siphon, which were completely transparent and free from pigment. Unfortunately, however, all attempts to repeat this experiment have been unsuccessful.

DISCUSSION

It has been shown that at least six members of the vitamin B complex are essential nutritive factors for mosquito larvae. Of these, five, namely, thiamin, riboflavin, pyridoxin, nicotinic acid and pantothenic acid, appear to be necessary also for other insects, such as *Tenebrio molitor* larvae (Martin & Hare, 1942), *Drosophila melanogaster* (Tatum, 1941), *Tribolium confusum* and *Ptinus tectus* (Fraenkel & Blewett, 1943).

The evidence provided above is the first indication that folic acid plays a part in insect nutrition. Such an observation might have been anticipated from the widespread activity of this factor in various organisms such as bacteria, chicks, trout and monkeys. The role of folic acid in the case of mosquito larvae is equally fundamental in importance, since the fourth-instar larvae cannot pupate in the absence of this factor.

From the work of Briggs, Luckey, Elvehjem & Hart (1944) it would appear that the concentrates used by us as sources of folic acid may also contain vitamins B_{10} and B_{11}. Thus while it is probable that the active factor in our preparations is folic acid, it

Table 6. *Effect of transferring larvae to and from folic acid-free media*

Larvae transferred immediately after reaching stage of development indicated by heavy line.
No. = number in stage of development indicated.
Mean, S.D. = mean and standard deviation of time (in days) taken to change from the previous stage.

Larval instars							Pupae		Adults		Total to adult
I	II		III		IV						
No.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	No.	Mean S.D.	Mean S.D.
Larvae transferred to folic acid medium											
17	17	1·9±0·49	17	1·6±0·53	17	1·3±0·35	15	3·1±0·67	15	2·1±0·28	9·8±1·55
20	20	1·8±0·40	20	1·7±0·42	20	2·3±0·37	19	3·0±0·55	19	2·2±0·26	10·9±0·99
13	13	2·0±0·51	13	2·0±0·60	13	5·5±2·25	5	8·9±6·37	2	3·5 —	16 —
Larvae transferred to folic acid-free medium											
15	15	1·7±0·25	15	1·3±0·47	15	1·8±0·48	8	5·8±2·57	4	2·1±0·22	10·4±0·89
17	17	1·8±0·46	17	1·1±0·29	17	1·4±0·76	14	3·1±1·29	12	2·4±0·19	9·4±1·19
18	18	1·6±0·28	18	1·3±0·58	18	1·4±0·47	18	2·9±0·99	15	2·2±0·36	9·2±2·17
Control larvae in folic acid-free medium											
40	37	2·2±0·63	37	2·5±1·11	24	6·4±3·15	0	—	—	—	—

cannot be regarded as certain. Whatever its real identity, this factor must be fairly specific in its action, for xanthopterin and thymine, both substances which replace folic acid in certain cases, are incapable of doing so in this instance.

It is possible to look upon folic acid as but one of a number of factors in the absence of any one of which pupation will not occur. It is our intention to investigate the effects of other vitamin deficiencies in the presence of folic acid when the pure substance becomes available. Even at the present juncture, however, it can be pointed out that lack of any of the other essential vitamins does not permit growth to the fourth stage; indeed, its effect is usually clear even in the second instar. It is only lack of folic acid which exercises such a dramatic effect at one stage of development.

The mechanism by which folic acid induces pupation remains to be elucidated. The transference experiments recorded above make it appear unlikely that folic acid is itself a pupation hormone. It is more probable that it functions by stimulating the production of a pupation hormone within the growing larva but that it can no longer exercise this effect in a fully grown larva. The crucial period appears to be the third larval instar.

SUMMARY

1. Autolysis products of fresh brewer's yeast, tested for their ability to promote growth of larvae of *Aedes aegypti*, revealed the loss of an essential part of the activity of the original yeast. Under suitably modified conditions this loss could be avoided.

2. A basal medium comprising the water-insoluble factor of brewer's yeast, glucose, salt mixture and yeast nucleic acid requires supplementation with thiamin, riboflavin, nicotinic acid, pantothenic acid and possibly with biotin in order to permit larval growth to the fourth instar.

3. For pupation, one further factor appears to be necessary, namely, folic acid. Its effect is specific, for it cannot be replaced by xanthopterin or thymine. It exercises an important effect also on growth and survival rates, bodily pigmentation and size of the larvae.

4. The following substances when added to or withheld from various media did not appear to influence growth or survival of the larvae; other vitamins of the B group, amino-acids, purines, pyrimidines, glutathione, ascorbic acid and possibly vitamin K.

5. Transference of larvae from a folic acid-free medium to one containing the vitamin, and vice versa, revealed that the presence of folic acid seems to have its most vital effect during the third stage of larval life.

We are indebted to Misses L. O'C. Biack and S. Sussman for technical assistance. Our thanks are due to the Castle Breweries, Johannesburg, for supplies of brewer's yeast; to Prof. R. J. Williams for providing us with folic acid concentrate; to Lederle Laboratories Inc., for a sample of 'Norite eluate factor'; to Eli Lilly and Co. for a gift of biotin; and to Roche Products Ltd. for a gift of 'Synkavit'.

We wish to thank the Director of Pathology, S.A.M.C., who enabled one of us (M.L.) to participate in this work.

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TRANSPIRATION THROUGH THE CUTICLE OF INSECTS

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(*Received* 14 *November* 1944)

(With Plates 1 and 2 and six Text-figures)

The cuticle of most insects is an effective barrier to the evaporation of water. Since the work of Kühnelt (1928, 1939) it has been known that this impermeability is a property of the outermost layer. It is usually ascribed to the epicuticle—defined as the layer, of the order of 1 μ in thickness, that is not penetrated by the pore canals, contains no chitin, and resists solution in cold concentrated hydrochloric or sulphuric acids. From chemical tests Kühnelt concluded that the epicuticle contained fatty acids and cholesterol-like bodies. He therefore refers to it as a 'lipoid cuticula'.

The presence of lipoid material or of waxes in the outer parts of the insect cuticle has been indicated further by Wigglesworth (1933), Bergmann (1938), Pryor (1940), Hurst (1940) and others. But it has never been clear whether such materials were to be regarded as impregnating the lipoid-free substance of the epicuticle, as being chemically condensed or polymerized to compose the epicuticle or as forming a discrete layer upon its outer surface.

It was shown by Ramsay (1935) that the cockroach owes its impermeability to water to a thin and apparently mobile layer of lipoid on its surface. At a critical temperature of about 30° C. this lipoid seems to undergo a change of phase, and it then allows water to pass freely through it. This interesting observation has never been confirmed on other insects. It forms the starting-point of the present study.

PART I. THE EFFECT OF TEMPERATURE ON TRANSPIRATION

Material and methods

Representative insects of diverse groups from different habitats have been exposed for short periods in dry air and their loss of weight by evaporation measured at different temperatures. In most experiments the insects have been first killed with hydrogen cyanide and the spiracles occluded with cellulose paint. They have been placed in a small basket of metal gauze, usually three or four together, and suspended in a conical flask over phosphorus pentoxide. The flask is immersed in a water bath and the temperature recorded on a thermometer with the bulb close beside the suspended basket. The small opening to the flask, combined with the large surface of the desiccating agent and the relatively small air space, ensure the maintenance of an almost dry atmosphere around the insect

When the same insects are used the same rates of evaporation are obtained whether they are exposed in this apparatus for 15, 30 or 60 min. Equilibrium is probably established very rapidly. In most experiments the short exposures have been employed in order that the entire series of readings may be taken without depleting the water content of the insect unduly. Provided the spiracles are sealed, there is no difference in the rate of evaporation from dead or living insects.

The rate of loss has been expressed in mg. per sq. cm. of surface per hour. The estimation of the effective 'surface' of the insect is a difficult matter. Where the rate of passage of water is very slow every little convolution and irregularity in the surface ought probably to be taken into account. Where the permeability of the cuticle is greater, the concentration of water vapour in the finer irregularities will make the gross surface a truer measure of the effective evaporating area. For the present purpose the fine foldings of the surface have been disregarded. The cuticle of an insect of known weight has been cut up and its surface area measured on squared paper. From this figure the value of k in the formula $S = k \times W^{\frac{2}{3}}$ has been found and so the surface area in sq. mm. (S) of any other member of the same species calculated from the weight in mg. (W). The following are examples of the values of k that have been employed: *Agriotes* larva, 11·0; *Tenebrio* larva, 8·4; *Calliphora* larva, 6·8; *Nematus* larva, 8·5; *Pieris* larva, 9·8; pupa, 8·4; *Rhodnius* nymph (recently fed), 8·1. This procedure gives only approximate values; but the specific differences in evaporation are so great that an error of 50% in the value of k will not affect the conclusions drawn.

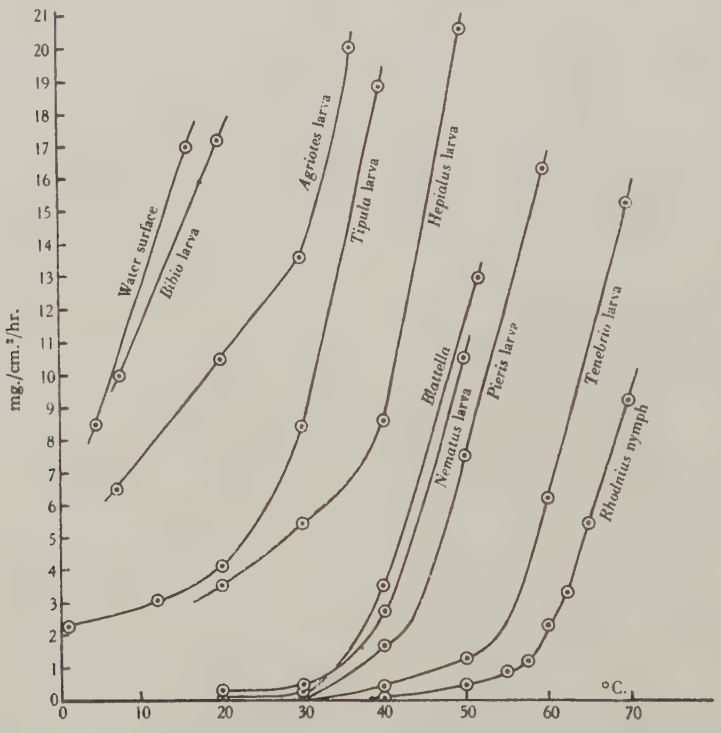
The rate of evaporation from a free-water surface was obtained by exposing a shallow capsule brimful of water in the same apparatus.

Results

Text-fig. 1 shows the results obtained on a small series of insects representative of different habitats. Each point represents a mean value from four or

eight individuals. The curve for water loss in *Blattella germanica* agrees fairly well with that obtained by Ramsay (1935) on *Periplaneta*; it shows a 'critical temperature', with an abrupt increase in evaporation a little above 30° C. The curve of the leaf-eating larvae of the sawfly *Nematus ribesii* and the caterpillar *Pieris brassicae* do not differ greatly from that of *Blattella*. But the insects from dry environments, which can survive long periods without feeding (larvae of *Tenebrio molitor* and nymphs of *Rhodnius prolixus*) show a critical temperature 20–30° C. *oleracea* and *Hepialus lupulinus*, the rate of evaporation at low temperatures is less and there is evidence of a rather ill-defined critical temperature.

Differences in the rate of transpiration through the cuticle and in the critical temperature may be well shown in the different stages of a given insect. This is illustrated in Text-fig. 2. In *Tenebrio* there is little difference in the transpiration curves of larva, pupa or adult. But there is a great increase in the waterproofing of *Bibio* on pupation; and in the pupa of *Pieris*, which has to withstand exposure in the open

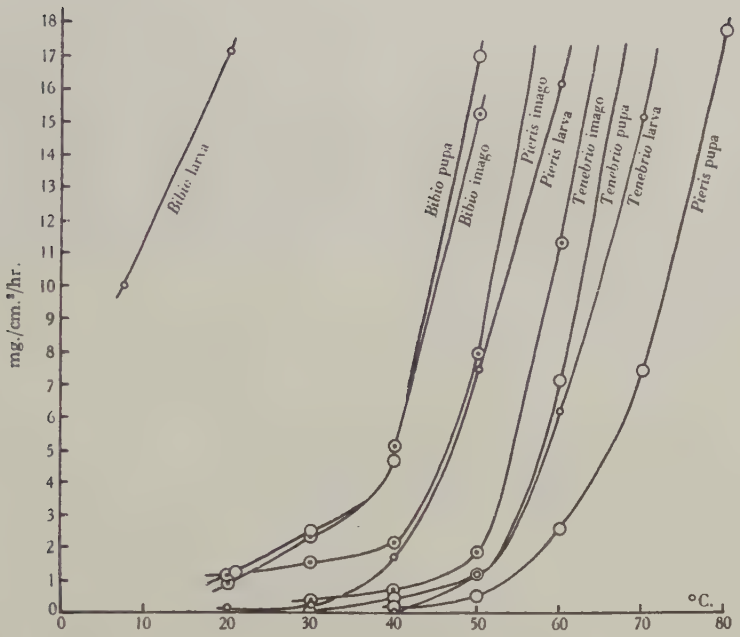


Text-fig. 1. The rate of evaporation of water from dead insects at different temperatures.

higher. Below the critical temperature the transpiration through the cuticle is very small in all these insects, though it is noteworthy that at say 20° C. the rate is generally higher in the insects with the lower critical temperature.

On the other hand, the group of insects from the soil, which normally exist in an atmosphere saturated with moisture, all show a high rate of transpiration even at the lowest temperatures. In the larvae of *Bibio marci* the rate approximates to that of a free-water surface, with no visible break in the curve. In the larvae of wireworms *Agriotes* spp., of *Tipula* for many months, the critical temperature exceeds even that of *Tenebrio* and *Rhodnius*. In these experiments the spiracles of the imagines of *Bibio*, *Tenebrio* and *Pieris* were not blocked. The true curve of evaporation for the adults should therefore probably lie slightly to the right.

These and other results are summarized in Table 1, in which the standard of comparison used is the temperature at which the loss of weight by evaporation amounts to 5 mg. per sq. cm. of surface per hour. This figure has been read off the evaporation curves; it gives a value perhaps 8 or 10° C. above the



Text-fig. 2. The rate of evaporation of water from dead insects in larval, pupal and adult stages at different temperatures.

Table 1. *Temperature in ° C. at which evaporation into dry air equals 5 mg. per sq. cm. per hr.*

Insect	Larva	Pupa	Imago
Bibio marci (Dipt. Bibionidae)	< 5	39·5*	40·5*
Pterostichus madidus (Col. Carabidae)	< 10	40·5*	40·0*
Agriotes sp. (Col. Elateridae)	< 10	44·5*	46·0*
Aphodius fimitarius (Col. Scarabaeidae)	15·5	—	—
Agrotis segetum (Lep. Noctuidae)	19·0	63·5	—
Tipula oleracea (Dipt. Tipulidae)	23·0	62·0	—
Phyllopertha horticola (Col. Scarabaeidae)	25·5	34·0	45·0*
Hepialus lupulinus (Lep. Hepialidae)	28·0	42·0	—
Blattella germanica (Orth. Blattidae)	—	—	42·5
Calliphora erythrocephala (Dipt. Muscidae)	41·0	57·0	43·5*
,, ,, (prepupa)	41·0	—	—
,, ,, (puparium)	41·0 (65·0)†	—	—
Nematus ribesii (Hym. Tenthredinidae)	43·5	—	—
Pieris brassicae (Lep. Pieridae)	46·5	66·5	46·0*
P. rapae (Lep. Pieridae)	48·0	65·0	—
Ephestia kuehniella (Lep. Pyralidae)	57·5	61·5	47·0*
Achroia grisella (Lep. Pyralidae)	59·5	57·0	—
Tenebrio molitor (Col. Tenebrionidae)	58·5	63·0	54·0*
Rhodnius prolixus (Hem. Triatomidae)	64·5	—	—

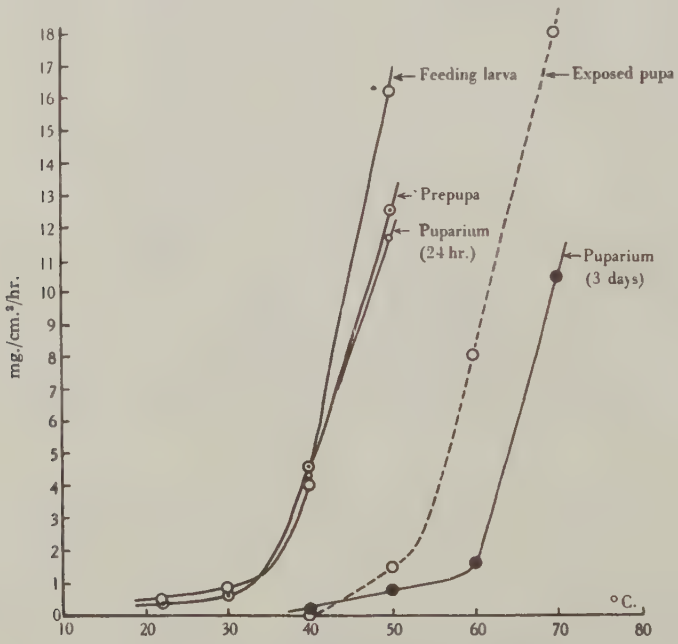
* Spiracles not blocked. † Three days old.

critical temperature, which is itself difficult to define without taking measurements at a large number of temperatures.

Evaporation from Rhodnius prolixus

The foregoing comparative results have been based on measurements of evaporation at a few temperatures only, usually at intervals of 10° C. More detailed studies were made on nymphs of *Rhodnius prolixus*. 4th stage and 5th stage nymphs 1 or 2 days after feeding have been used; they have identical curves of evaporation. As shown in Text-fig. 1, measurements were made at intervals of 2·5° C.

On the other hand, this constancy is not altogether maintained if the insect is exposed alternately to high and low temperatures. After exposure to temperatures above the critical level there is some slight permanent increase in transpiration on return to a lower temperature; and this increase is greater the higher the temperature has been. Thus in successive exposures for 15 min. the loss of weight in mg. per hour in one group of 5th-stage nymphs was as follows: 50° C. 0·9; 65° C. 12·4; 50° C. 2·2; 80° C. 56·8; 50° C. 3·8. In another group the rates of loss were as follows: 50° C. 1·4; 80° C. 58·0; 50° C. 3·8; 50° C. (after keeping 24 hr.) 3·2; 50° C. (after keeping a further 3 days) 3·8. The exposure to high temperatures has clearly done some slight permanent injury to the water-proofing layer.



Text-fig. 3. The rate of evaporation of water from *Calliphora* in larval and pupal stages at different temperatures.

around the critical temperature, each point being based on the averages of from four to twelve insects. It can be seen that there is an abrupt change in the rate of transpiration at about 57·5° C.; but even at 70° C. the evaporation is not more than one-sixth of that from a free-water surface at that temperature.

The rate of transpiration at a given temperature seems to be a definite figure which remains constant on repetition. Thus in a group of three 5th-stage nymphs with spiracles blocked the loss of weight in mg. per hour at 60° C. in four successive exposures of 15 min. was: 2·4, 2·8, 2·0, 2·8. The same insects exposed at 70° C. gave losses per hour in three successive exposures of 18·6, 17·6 and 17·6 mg.

Evaporation from larvae and pupae of Calliphora erythrocephala

The results obtained with larvae and pupae of *Calliphora* are set out in Text-fig. 3. The insects have been killed with cyanide and the spiracles blocked. Feeding larvae removed from the meat, larvae (so-called 'prepupae') which have left the meat and evacuated the gut, and fully darkened puparia 24 hr. old, all show curves for evaporation that are substantially alike.

On the other hand, there is a very striking displacement of the curve, with a great reduction in evaporation at high temperatures, in puparia 3 days old in which pupation has occurred. This increased water-proofing is due, not to any change in the puparium, but to the delicate cuticle of the true pupa; for when the puparial shell is peeled off and the young pupa exposed, there is only a relatively slight increase in permeability at high temperatures —such as would result from the removal of a comparatively permeable outer sheath.

The pupal cuticle in *Calliphora* is excessively fragile. These observations therefore afford further evidence of the importance of the thinnest layers in the prevention of evaporation.

Evaporation from larvae of Agriotes

Each point on the curve for evaporation from *Agriotes* larvae as given in Text-fig. 1 was derived from a separate group of larvae. For it was found that in these larvae the rate of evaporation at a given temperature diminished at each successive exposure. Thus a larva weighing 37·6 mg. was exposed for six successive periods of 15 min. at 30° C. The rates of loss expressed in mg. per sq.cm. per hour were as follows: 17·2, 11·6, 9·6, 7·6, 4·4, 2·8. The low final value may have been influenced by the depletion of water in the body; but the rapid initial fall must be due to a progressive reduction in the permeability of the cuticle*. A similar effect was seen in some of the *Aphodius* larvae; but the phenomenon has not been more closely studied.

The significance of the great and varied permeability of the insects living in the soil will be discussed further when the action of abrasive dusts has been considered. Further results on evaporation from the wireworm will then be given (p. 106 and Text-fig. 6).

PART II. THE EFFECT OF ABRASIVE DUSTS ON TRANSPIRATION†

It has been known for many years that insects in dry environments may be killed by exposure to certain chemically inert dusts, and it has been generally agreed that the cause of death is desiccation. The subject has been fully reviewed recently by Alexander, Kitchener & Briscoe (1944). These authors (and cf. Parkin, 1944) have made an extensive comparative study of the action of such dusts and have proved conclusively that they act by increasing the rate of transpiration through the cuticle. They suggest that this may be due to the 'crystalline forces' at the surface of the particles of dust exerting a specific attraction on the fatty film of the epicuticle and thus interrupting the continuity of the water-proofing layer.

In the present experiments the chief dust used has been alumina, which Alexander *et al.* (1944) have shown to be one of the most active, but some comparative observations have been made with powdered quartz and slate dusts.

Effect of dusts on evaporation in Rhodnius

Alexander *et al.* (1944) found that the dusts had no action on dead insects. This has been readily confirmed in *Rhodnius*. 5th stage nymphs of *Rhodnius* 1 day after feeding, with an average weight of 132 mg., were killed with cyanide and the spiracles sealed with cellulose paint. They were then kept at 30° C. over phosphorus pentoxide for 24 hr., half being thickly dusted with alumina and half serving as controls. The controls lost an average of 2·0 mg. (1·5 % of the weight), the insects covered with alumina lost 1·7 mg. (1·3 %).

Similar insects, with the anus blocked with paraffin, allowed to run on filter paper lightly dusted with alumina under the same conditions were completely shrunken and dead in 24 hr., having lost 46·5 % of their weight. Insects running on clean filter paper lost only 2·2 %.

But the living state of the insect does not appear to be the determining factor. Living 5th stage nymphs with normal spiracles were suspended in mid-air by sealing the thorax to the head of a bent pin with paraffin and sealing the point of the pin to the wall of a small capsule (Text-fig. 4A). Control insects suspended in dry air at 30° C. lost 1·8 % of their weight in 24 hr. Insects heavily dusted with alumina all over the body, including the spiracles, lost 1·9 %.

The insects suspended in this manner remain motionless during the experiment. It seemed likely that it is the movement of the insect which is needed for the action of the dust. It was observed that as the engorged *Rhodnius* nymph crawls on a surface the posterior region of the ventral abdomen touches the ground and is continually rubbed (Text-fig. 4B). If a small amount of paraffin wax is placed on this bearing surface so that it is held away from the ground (Text-fig. 4C) and the insect is then allowed to run on the dusted filter paper, it suffers comparatively little desiccation and instead of dying within 24 hr. in dry air it will survive for days. And this is so in spite of the fact that the whole of the body soon becomes covered with a film of dust.

Experiments along these lines, using alumina, finely powdered quartz (with particles of 0·5–1·0 μ)* and fine slate dust† are summarized in Table 2. As seen in the same table, a considerable increase in evaporation occurs if the insect runs on fine emery cloth.

* Dr D. L. Gunn has pointed out to me that a similar decrease in permeability to water following desiccation is seen in non-living membranes (cf. King, 1944).

† Certain of these results have been published in a preliminary note (Wigglesworth, 1944*a*). Confirmatory observations were reported by Kalmus (1944).

* Kindly supplied by Dr G. Nagelschmidt.

† Kindly supplied by Dr I. Thomas.

These experiments suggest that the dusts act as simple abrasives and that they increase the permeability of the cuticle only if this is rubbed upon the dusted surface. As seen in Table 2, there is some increase in evaporation even when the bearing surface is protected by wax. As will be shown later

Table 2. *Average percentage loss of weight in 24 hr. in dry air at 30° C. from recently fed 5th stage nymphs of* Rhodnius

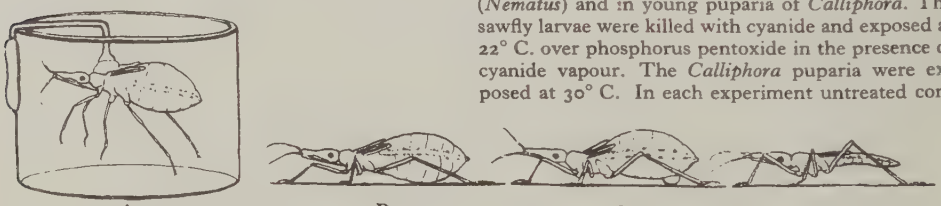
Running on clean filter paper	2·1
Running on emery cloth no. 0	7·8
Running on filter paper dusted with slate dust	8·4
Running on filter paper dusted with slate dust with mound of wax on the bearing surface	4·3
Running on filter paper dusted with fine quartz dust	17·8
Running on filter paper dusted with fine quartz dust with mound of wax on the bearing surface	5·2
Running on filter paper dusted with alumina	46·5
Running on filter paper dusted with alumina with mound of wax on the bearing surface	7·6

demonstrated on the dead insect. Dead 4th-stage nymphs, recently fed, and with the spiracles blocked, in dry air at 30° C. for 5 hr. lost 0·8 % of their initial weight. Similar insects pressed firmly down into a thick layer of alumina lost 0·9 %. Insects pressed lightly against dusted filter paper and at the same time drawn along, lost 30·0 %. In this last experiment the dust was removed from the cuticle within less than a minute of application by brushing with a camel's-hair brush under a stream of water.

Effect of dusts on evaporation in some other insects

It is clear that the impermeable film on the surface of *Rhodnius* is interrupted only by the mechanical action of the dusts. Dust in stationary contact with the cuticle will not adsorb lipoid material and so break the film. But the cuticular wax of *Rhodnius* is a hard material with a high melting-point (Beament, 1945) and a high critical temperature. It is possible that adsorption alone may serve to remove the waterproofing layer in insects with waxes of lower melting-point.

This has been tested in larvae of the sawfly (*Nematus*) and in young puparia of *Calliphora*. The sawfly larvae were killed with cyanide and exposed at 22° C. over phosphorus pentoxide in the presence of cyanide vapour. The *Calliphora* puparia were exposed at 30° C. In each experiment untreated con-



Text-fig. 4. A, method of suspending 5th stage nymph of *Rhodnius*. B, 5th stage nymph of *Rhodnius*, fed; showing how the abdomen rubs against the ground. C, the same; abdomen held away from the ground by a mound of paraffin. D, 5th stage nymph, unfed; abdomen out of contact with the ground.

(p. 103), this is due to the chance abrasion of other parts and to the dust getting between the moving surfaces of the limb joints and causing their abrasion. A similar difference can be seen when fed and unfed nymphs are allowed to run on dusted filter paper. In the unfed insect the abdomen is held above the surface and only rubs against it occasionally (Text-fig. 4D). In one such experiment, during 24 hr. in dry air at 30° C., the loss of weight was as follows:

Fed insects on clean filter paper	6·5 mg.
Fed insects on dusted filter paper (alumina)	47·7 mg.
Unfed insects on clean filter paper	0·4 mg.
Unfed insects on dusted filter paper (alumina)	7·0 mg.

As can be seen in Table 2, the quartz dust is less effective than the alumina, and the slate dust less effective still. A difference can be readily appreciated by the tip of the finger drawn across the dusted paper. That treated with alumina feels like exceedingly fine sandpaper; the quartz dust feels less abrasive; the slate dust smoother still.

The necessity for abrasion by the dust can be

Table 3

Insect and treatment	Loss of weight % in 24 hr.
Nematus larva	
A. Control	8·2
B. Rubbed with alumina	66·5
C. Sprinkled with alumina	8·3
Calliphora puparium 24 hr. old	
A. Control	14·6
B. Rubbed with alumina	51·0
C. Sprinkled with alumina	15·0
Calliphora puparium 3–4 days old	
A. Control	4·5
B. Rubbed with alumina	4·0
C. Puparial shell largely removed	6·9

trols were compared with insects rubbed on filter paper dusted with alumina and with insects sprinkled with the dust. The results (Table 3) show that in

these insects, as in *Rhodnius*, rubbing with the dust increased greatly the rate of evaporation but that sprinkling with the dust had no action. In the puparium of *Calliphora* 3–4 days old, in which pupation has taken place, rubbing with the dust naturally has no effect.

In the cockroach, as was shown by Ramsay (1935), the waterproofing lipoid is far more mobile; for example, it will spread over the surface of water droplets sprayed on to the cuticle and protect them from evaporation. Table 4 shows the results of an experiment with the cockroach (female *Blatta*), killed with cyanide and exposed to dry air in the presence of cyanide. Control insects (A) were compared with insects (B) in which the dorsal and ventral surfaces of the abdomen were rubbed on filter paper dusted with alumina and the dust then wiped off, and with others (C) sprinkled all over with alumina. It can be seen that in the cockroach simple dusting without rubbing causes rapid desiccation. This is apparent within the first 4 hr. after dusting. During this period the rate of water loss was greater in the insects which had been rubbed with the dust; but during the next 20 hr., whereas the rate of loss from the rubbed insects had diminished, the loss from the insects sprinkled with the dust had increased. During the ensuing 24 hr. the rubbed insects showed a further diminution; the sprinkled group would probably have lost water at a still greater rate but for the fact that they had become completely dried up.

Table 4. *Evaporation from dead cockroach* (Blatta) *at* 20° C. *in dry air expressed as mg. per hr. during successive periods of exposure. Average initial weight:* 880 *mg.*

Treatment	First 4 hr.	Next 20 hr.	Next 24 hr.	Total loss %
A. Control insects	1·9	1·5	1·4	8·4
B. Tergites and sternites of abdomen rubbed with alumina	9·7	5·2	3·4	32·0
C. Sprinkled with alumina	6·6	9·9	(7·2)*	46·8

* Almost fully desiccated.

Visible effects of dust on the cuticle

The cuticle of *Rhodnius*, where it has been rubbed with alumina by allowing the 5th stage nymph to crawl on dusted filter paper or by pressing the insect against the filter paper and drawing it along, has been examined microscopically in surface view and in sections. No injury to the epicuticle or to the bristles can be detected. The layer which prevents evaporation probably lies, therefore, on the outer surface of the epicuticle.

Now it has been shown by Schmallfuss and others (1933) that the outer layers of the insect cuticle commonly contain dihydroxyphenyl-alanine, dihydroxyphenyl-acetic acid or other polyphenols which provide the chromogens used in the production of melanin; and Pryor (1940) has found evidence to show that the quinones produced by oxidation of the polyphenols, by 'tanning' the cuticular proteins, are responsible for the hardening of the cuticle after moulting. The polyphenols are readily demonstrated by exposure to ammoniacal silver hydroxide, which they reduce to give a deep brown stain (Lison, 1936). Pl. 1, fig. 3, shows a section of a fresh *Rhodnius* cuticle, cut with the freezing microtome and immersed for 1 hr. in ammoniacal silver hydroxide. The epicuticle is everywhere stained dark brown.

If the living *Rhodnius* nymph, however, is immersed in freshly prepared 5% ammoniacal silver hydroxide, washed in distilled water, and the cuticle then dissected off, fixed in Carnoy's fixative and mounted flat, there is no sign of staining. The polyphenols are clearly separated from the solution by a protective layer (Pl. 1, fig. 2). But if the insect has been rubbed with alumina, or allowed to crawl on filter paper dusted with alumina or on fine emery cloth, there is very obvious brown staining over the rubbed areas. This staining is confined to the prominent points of the cuticle: the domes of the raised plaques from which the bristles arise and the crests of the stellate folds into which the epicuticle is thrown (Pl. 1, figs. 1, 4). The protective layer has clearly been rubbed off these points.

When the insect has been crawling on dusted filter paper there are brown staining patches also where the tibia and tarsus have been drawn across the surface, and between the articulations of the limb joints where these have rubbed together. The insects that have crawled on emery cloth often suffer a more brutal abrasion and obvious scratches can be seen (Pl. 1, fig. 5).

In section the brown staining of the prominent crests is seen to be confined to the epicuticle (Pl. 1, fig. 4). Nymphs rubbed with the dust have been immersed in the silver solution for periods ranging from 5 min. to 2 hr. The prolonged exposure causes only a slight increase in the extent of the brown staining areas. These spots therefore probably represent the true limits of the areas from which the protective layer has been abraded.

Similar results have been obtained with other insects. *Calliphora* puparia show no staining on immersion in the silver solution; but after rubbing gently on the dust, brown staining spots appear on all the prominent ridges (Pl. 1, fig. 6). Larvae of *Tenebrio* kept for a few hours on dusted filter paper show brown staining areas where the coxae rub against the thorax (Pl. 1, fig. 7), between the limb joints, and at all those points on the ventral surface of the abdomen where this touches the ground as the larva creeps along. Larvae of *Ephestia* on dusted filter paper are very rapidly affected and become desiccated within a few hours. Treatment with silver shows that not only has the protective layer been abraded from the

prolegs and wherever the cuticle has touched the ground, but the dust has also got into the surface folds everywhere so that staining occurs wherever one point on the surface rubs against another. In general, the rate of water loss from an insect treated with the dust runs parallel with the extent of the abraded areas as shown by treatment with silver.

Recovery of impermeability after treatment with dust

5th stage nymphs of *Rhodnius* 24 hr. after feeding were treated with alumina by laying them on their backs on dusted filter paper and drawing them over the surface for a standard distance while pressing them gently down with the finger. In this way a rounded area of fairly constant extent on the dorsal surface is abraded. The dust was removed at once with a camel's-hair brush under a stream of water. The rate of evaporation in dry air at 30° C. was measured immediately after abrasion and after the insect had been kept at 25° C. in saturated air for several days. The results are shown in Table 5.

Table 5. *Loss of weight of 5th stage nymphs of* Rhodnius, *with mean initial weight of 120 mg., during 24 hr. in dry air at* 30° C.

A. Immediately after rubbing with alumina	31·1 mg.
B. After keeping 1 day in moist air at 25° C.	5·2
C. After keeping 2 days in moist air at 25° C.	3·4
D. After keeping 3 days in moist air at 25° C.	2·4
E. Normal controls	1·5

Similar recovery has been demonstrated in larvae of *Tenebrio* and in pupae of *Pieris brassicae* after rubbing with the dust (Table 6).

Table 6. *Loss of weight during 24 hr. in dry air at* 30° C.

1. Pupae of *Pieris brassicae* (mean initial weight 370 mg.):	
A. Immediately after rubbing with alumina	42·1 mg.
B. After keeping 2 days in moist air at 20° C.	8·6
C. After keeping 6 days in moist air at 20° C.	4·3
D. After keeping 2 weeks in moist air at 20° C.	3·2
E. Normal control	0·7
2. Larvae of *Tenebrio molitor* (mean initial weight 90 mg.):	
A. Immediately after rubbing with alumina	46·5 mg.
B. After keeping 4 days in moist air	5·5
C. Normal control	1·0

It is clear that to a large extent the impermeability of the cuticle can be restored, although recovery never seems to be complete. This recovery is dependent on the activity of the epidermal cells and takes place only if they are living. Thus 4th stage nymphs of *Rhodnius* were dusted by rubbing as described above and the dust removed immediately. Some of these were exposed to dry air at once; the remainder were kept in saturated air for 24 hr., half of them living and half of them after exposure to hydrogen cyanide. At the end of 24 hr. those exposed to cyanide appeared dead, but the gut was still showing peristaltic movements. Table 7 shows the evaporation from these three groups of insects.

Table 7. *Loss of weight of 4th stage nymphs of* Rhodnius, *with mean initial weight of 95 mg., during 24 hr. in dry air at* 30° C.

A. Alive: immediately after rubbing with alumina	36·1 mg.
B. Killed with HCN: immediately after rubbing with alumina	37·3
C. Alive: after keeping in moist air for 1 day	9·2
D. Killed with HCN: after keeping in moist air for 1 day	36·6

The importance of the living cells is shown also by experiments in which the cuticle has been abraded by dusting in the later stages of moulting, at a time when the old cuticle has become detached from the cells and the new cuticle is being laid down but has not yet become impermeable. The 5th stage nymph moults at about one month after feeding. Table 8 shows the absence of recovery in insects dusted at three weeks.

Table 8. *Percentage loss of weight of 5th stage nymphs rubbed with alumina and the dust removed at once, kept for 1 day at 25° C. in moist air and then exposed for 24 hr. in dry air at* 30° C.

A. 3 days after feeding	3·8
B. 3 weeks after feeding	31·9

We have seen that in *Rhodnius* and most other insects adsorption by the dust will not by itself break down the protective film. But once this film has been interrupted by abrasion the presence of the dust may seriously interfere with the recovery process. This may be shown by comparing the efficiency of recovery in insects in which the dust has been removed immediately after dusting, with others in which the dust has been left on (Table 9). When the dust is left on the cuticle, recovery is very incomplete even after many days.

Table 9. *Percentage loss of weight of 5th stage nymphs rubbed with alumina, exposed for 24 hr. in dry air at* 30° C.

A. Immediately after rubbing; dust left on	48·2
B. Immediately after rubbing; dust removed	47·5
C. After keeping in moist air for 2 days; dust left on	16·8
D. After keeping in moist air for 2 days; dust removed	3·1

In the cockroach, on the other hand, as shown in Table 4, adsorption by the dust quickly leads to increased evaporation. The high degree of mobility in the waterproofing grease of the cockroach not only leads to its removal by adsorption, but, as Table 4 also shows, it allows a considerable amount of recovery in the dead insect—by the spreading of the oil over the abraded areas.

Visible changes during recovery

If the dust has been removed from the *Rhodnius* nymph as completely as possible immediately after application, and the insect then kept in moist air for a few days, the rubbed cuticle develops a milky bloom. The effect is more striking if the dust is left on; what is at first an almost imperceptible film is then converted within 2 or 3 days into a conspicuous chalky white patch, more or less firmly adherent to the surface of the cuticle. In both cases this waxy secretion is more evident if the insect after recovery is kept in a dry atmosphere, just as certain plants (which likewise are able to regenerate their waxy coating) produce less wax if they are grown in a damp atmosphere (Haberlandt, 1914).

As the new wax is secreted the cuticle becomes less readily wetted by water. At first droplets of water will adhere to the surface; after a few days the droplets fall from the cuticle without wetting it. Measurements of the angle of contact between water and the cuticle (kindly made for me by Mr Beament) show that this increases from 102° to nearly 120° during the first 48 hr. as the wax is being secreted. It is of interest to note that the angle increases also, though more slowly, on the normal cuticle as moulting proceeds; by 21 days after feeding it has increased gradually from 102° to 117°. An extra quantity of wax thus appears to be secreted on to the surface of the old cuticle before the epidermal cells separate from it and so lose the power of making good any abrasion that may occur.

The presence of the wax may be demonstrated also with fat stains. If the cuticle of the insect which has recovered from dusting is heated with Black Sudan B at 50° C., the waxy deposits, chiefly on the sides of the epicuticular folds, are deeply stained. This staining is very much more conspicuous if the dust has not been removed (Pl. 1, fig. 10).

As the wax is secreted, and the impermeability of the cuticle is restored, the patches that stain with silver become smaller; and after recovery has proceeded for a few days no reduction takes place on immersion in the silver solution. Pl. 1, figs. 8 and 9 show two stages in this process. From the way in which the individual star-shaped areas on the crests of the folds are broken up it is evident that the secretion which covers them is coming through the substance of the cuticle everywhere. It clearly does not spread out from the openings of the ducts of the dermal glands; it must be the product of the general epidermal cells.

During the process of recovery after abrasion there are striking changes also in the underlying epidermal cells. In spite of the fact that there is no visible injury to the epicuticle the underlying cells behave as though they had been wounded (Wigglesworth, 1937). They migrate towards those regions that have been most severely rubbed, leaving areas with sparse cells between. This response is not the result of injury to the cells by desiccation, for it occurs equally in insects that have been kept in air saturated with moisture. Indeed, the cells behave as though their living substance extended not only by way of the pore canals up to the epicuticle, but through the epicuticle up to the protective film on its surface.

During the next day or so small droplets which stain with fat stains and reduce osmic acid appear in the epidermal cells most affected. This is probably a further reaction to injury; the droplets do not seem to be related with the secretion of wax that is occurring at this time. Another change is the failure of the epidermal cells under the rubbed area to develop the large amounts of red pigment or the white granules (believed to be uric acid) which they normally contain.* At no stage is there any sign of renewed activity in the dermal glands.

Natural abrasion of the cuticle in soil insects

It is evident from Text-fig. 1 that the larvae of insects from the soil lose water very readily at quite low temperatures. The question naturally arises whether this is the result of abrasion by particles of earth. Immersion of the larvae in ammoniacal silver hydroxide reveals in all of them extensive areas over which the layer of polyphenols has been exposed, and in most of them these areas are made up of obvious scratches.

In *Hepialus*, after immersion in the silver solution, there are dark brown areas over all the projecting folds of the body, particularly towards the anterior end (Text-fig. 5A). On microscopic examination these areas are seen to be made up of scratches crisscrossing in all directions. Scratches are very evident also on the outer surface of the true legs (Pl. 1, fig. 11). In *Agrotis* the results are the same. It is noteworthy that in both these caterpillars the cuticle is much more resistant to scratching over the tubercles and over the head where there is a hard exocuticle. In *Aphodius* and *Phyllopertha* similar results are obtained: reduction of the silver appears in the form of scratches on the prominent lateral folds, on the anal extremity and the legs, but particularly over the surface of the rounded back (Text-fig. 5B; Pl. 1, fig. 13). The scratching of the back is less evident in *Phyllopertha*, in which the protective spicules are more strongly developed.

In the wireworm *Agriotes* the body is covered

* There is also an interesting effect on growth. The moulting of the insect is in no way delayed by extensive abrasion of the epicuticle, provided desiccation is prevented. But the process which leads to the determination of new plaques is upset. The number of plaques on one-half of an abdominal tergite when the 4th instar nymph moults to a 5th instar increases from about 200 to 250 (Wigglesworth, 1940). In a nymph which had been rubbed on one side of the abdomen only there were 206 plaques on the rubbed side, 258 on the normal. Injury to the cells caused by rubbing the layers *outside* the epicuticle has prevented the determination of new plaques.

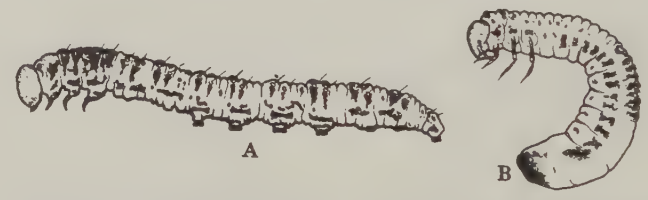
almost everywhere with hardened cuticle which resists extensive abrasion. But there are ventro-lateral lines of soft cuticle and some areas of soft cuticle at the base of the limbs which always show extensive browning in the silver (Pl. 1, fig. 12). And when examined with a higher magnification the hardened cuticle everywhere shows innumerable fine scratches. The degree of this visible scratching runs parallel with the rate of evaporation. Thus in one series of wireworms exposed at 40° C. the most permeable lost 39·2% of its weight in 15 min.; another lost only 17·2%. Pl. 2, figs. 1 and 2, show corresponding areas of cuticle from these two insects. The more permeable is much more severely scratched.

In the larva of *Pterostichus* the hard cuticle is restricted to plates or sclerites set like islands in the soft body surface (Pl. 2, fig. 3). Here the abrasion of the soft areas is so extensive that in many places they show a uniform brown staining with the silver, and individual scratches cannot be seen. But where there are depressed folds, as between the body segments, abrasion is incomplete and in some places linear scratches appear. These scratches are sometimes continuous with the scratches on the sclerites. On the sclerites, as on the hard parts of the other insects, abrasion is limited to fine scratches, running mostly in the long axis of the body (Pl. 2, fig. 4).

The larva of *Bibio* is everywhere covered with little plaques bearing rows of blunt spines. The plaques show some abrasion, and in the soft cuticle between them there are innumerable small areas which stain with the silver (Pl. 2, fig. 6). These areas do not take the form of scratches, but in view of the observations on the soft cuticle of other insects they are probably the result of abrasion. Likewise in the larva of *Tipula*, there are small scattered points over the cuticle which stain with the silver; in many places these lie in rows and are almost certainly due to abrasion (Pl. 2, fig. 5).

In all these insects the rate of evaporation of water at low temperatures runs parallel with the extent of the silver staining areas (Text-fig. 1). In *Bibio* and *Pterostichus* evaporation is so great that there is no sign of a critical temperature. In *Agriotes*, *Tipula*, *Hepialus*, etc., in spite of the high rate of water loss at the lowest temperatures, the curve turns steeply upwards as soon as the critical temperature is reached.

It is concluded therefore that the permeability of the cuticle in soil insects is the result of abrasion. If this is so, the insect which has moulted and been kept out of contact with the soil ought not to show any notable permeability to water until the critical temperature is reached. This has been tested on the wireworm. Larvae of *Agriotes* were placed singly in specimen tubes with a pledget of moist cotton-wool and a small piece of carrot. The date of moulting was noted and the larva was then kept for 2 weeks. Text-fig. 6 shows the temperature-evaporation curve for such a larva. It falls into line with that of insects from other environments: there is little evaporation until the critical temperature is reached. In an experiment done less than 24 hr. after moulting there was considerable evaporation at low temperatures and exposure to silver showed irregular diffuse areas of brown staining.



Text-fig. 5. Larva of *Hepialus* (A) and *Aphodius* (B) from the soil, after immersion in ammoniacal silver, showing the distribution of the chief areas where the wax layer has been abraded.

Abrasion of the cuticle and the entry of insecticides

Not only is the loss of water by evaporation increased after abrasion by rubbing with the dusts, but also the uptake of liquid water. For example, 5th stage nymphs of *Rhodnius* killed with cyanide 24 hr. after feeding and then immersed in distilled water for 24 hr. showed an average increase in weight (on an initial weight of about 120 mg.) of 0·4 mg. in the normal insect, and 4·1 mg. after rubbing gently on filter paper dusted with alumina. This observation falls into line with the osmotic uptake of water by normal (and therefore abraded) wireworm larvae living in the soil (Evans, 1943).

It was to be expected that this increased permeability of the cuticle would be reflected in an increased susceptibility to insecticides. This has been tested with nicotine and with rotenone. 5th stage nymphs of *Rhodnius* with uniformly thick cuticles were fed, and glass capsules of the type previously described (Wigglesworth, 1942) sealed to the dorsum of the abdomen with cellulose paint. A solution of 2% nicotine, in distilled water, was introduced into the capsule and the top sealed with a cover-glass. The normal insects were slightly affected in 6 hr.; they

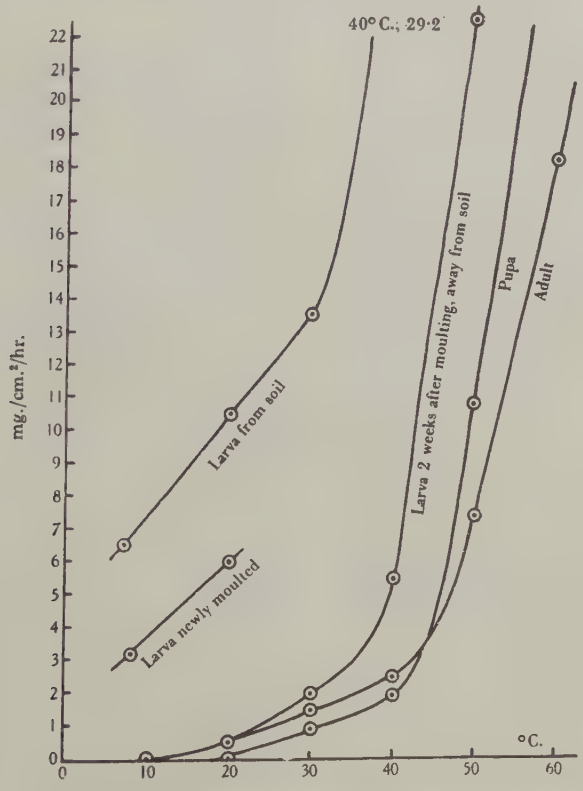
were badly affected but had not collapsed in 24 hr. Insects in which the back had first been lightly rubbed with alumina dust collapsed and were unable to walk within 20 min.

The experiment was repeated using dry powdered rotenone (Stafford Allen and Sons 90% rotenone) as the insecticide. The normal insects were unaffected at the end of 3 weeks; those rubbed with the dust showed weakness in 8 hr. and were dead in less than 24 hr.

PART III. THE EFFECT OF SOLVENTS AND DETERGENTS ON TRANSPIRATION THROUGH THE CUTICLE

Since oils and waxes are responsible for maintaining the impermeability of the insect cuticle to water, lipoid solvents and detergents should break down the protective layer.

Experiments along these lines have been made chiefly on *Rhodnius*. Chloroform was selected as a good general solvent for oils and waxes. Table 10



Text-fig. 6. Evaporation from the wireworm larva, pupa and adult at different temperatures.

These observations suggest that the activity of insecticidal powders may be enhanced if an abrasive dust is incorporated with the filler. Special studies will be needed to prove whether this happens in practice, but it is well known that the efficiency of a given insecticide is greatly influenced by the nature of the filler (Turner, 1943). Hockenyos (1939) noted that hydrated lime increased the rate of entry of sodium fluoride through the cuticle of the cockroach, and attributed this to an effect on the oil film.

Table 10. *Loss of water during 1 hr. in dry air at 30° C. from 5th stage nymphs of* Rhodnius 1 *day after feeding. Average initial weight 130 mg.*

A. Dead, normal controls	0·2 mg.
B. After extraction with chloroform for 15 min. at 20° C.	2·7
C. After extraction with chloroform for 15 min. at 50° C.	34·6

shows that there is a considerable increase in evaporation after extraction with chloroform at room temperature, a very great increase after extraction at 50° C. Insects from these three groups were subsequently immersed in ammoniacal silver hydroxide and the cuticle of the abdomen examined. The controls as usual showed no staining. Those extracted for 15 min. at room temperature showed staining over the muscle insertions (Pl. 2, fig. 7) but very little elsewhere. Those extracted for 15 min. at 50° C. showed well-marked staining over the muscle insertions and widespread staining elsewhere, particularly in the hollows of the epicuticular folds (Pl. 2, fig. 8).

The wax extracted with hot chloroform from the cast cuticle of *Rhodnius* is readily soluble in chloroform in the cold (Beament, 1945). The foregoing experiments show that extraction from the surface of the cuticle is very much more difficult, and that the ease of extraction varies in different regions. The structural basis of these observations will form the subject of a separate paper (Wigglesworth, 1944*c*); but the general conclusion may here be stated: that the wax layer overlying the epicuticle is further protected by a layer of cement which varies in hardness and resistance to removal in different insects and in different parts of the same insect.

The hardness of the wax in *Rhodnius* is such that the permeability of the insect is not very greatly affected if it is killed with chloroform vapour. But in the soft-skinned larva of the sawfly *Nematus*, and in the cockroach *Blatta*, exposure to the vapour of chloroform at room temperature for ½–1 hr. is sufficient to increase enormously the permeability of the cuticle to water (Table 11).

Table 11. *Loss of weight in* 4 *hr. at* 22° C. *of* Nematus *larvae with average initial weight of* 80 *mg., and* Blatta *adults with average initial weight of* 850 *mg., and in* 24 *hr. at* 30° C. *of* Rhodnius *nymphs, with average initial weight of* 110 *mg.*

Nematus larvae killed with HCN	1·0 mg.
Nematus larvae exposed to chloroform vapour for 1 hr.	39·3
Blatta adult females, killed with HCN	7·6
Blatta adult females, exposed to chloroform vapour for 1 hr.	49·7
Rhodnius nymphs killed with HCN	1·4
Rhodnius nymphs exposed to chloroform vapour for 1 hr.	3·8

We have seen (p. 105) that after abrasion with alumina dust the insect kept in a moist atmosphere will, to a great extent, recover its impermeability to water, and the points where the layer containing phenols was exposed once more become covered by wax. This recovered area does not resist extraction with chloroform as does the normal cuticle. Thus 5th stage nymphs of *Rhodnius* were rubbed with alumina on one-half of the abdomen only and then allowed to recover for 5 days in moist air. On treatment with ammoniacal silver after this period they showed no staining on either side. But after immersion in chloroform for ½ hr. at room temperature they showed on the normal side some staining over the muscle insertions and in the form of scattered points in the depressions between the cuticular folds. On the side that had been rubbed there was in addition extensive staining over the crests of the folds—though this staining was not quite so widespread as in the newly rubbed insect.

The same effect is apparent if the rate of evaporation after extraction with chloroform is observed (Table 12).

Table 12. *Loss of weight in mg. in* 4 *hr. at* 30° C. *in dry air from* 5*th stage nymphs of* Rhodnius 4 *days after feeding* (*average weight* 120 *mg.*)

A. Control	0·7 mg.
B. The same extracted with chloroform for 15 min. at 20° C.	6·9
C. 3 days after rubbing with alumina	1·4
D. The same extracted with chloroform for 15 min. at 20° C.	17·1

From these results it appears that the wax in the layer which overlies the epicuticle of *Rhodnius* is covered with or associated with materials which protect it from extraction. If the protective layer is to be broken down and the insect rendered permeable to water (and perhaps to insecticides) an agent must be used that will attack both the wax and the cement substance and at the same time be sufficiently hydrophil for water to pass readily through it.

With this end in view a long series of wetting agents and detergents and other chemicals have been tested. These have been applied as a thin smear over the dorsal surface of the abdomen of living 4th stage nymphs of *Rhodnius* 24 hr. after feeding. Groups of three or four nymphs have been used for each compound and with many of the compounds the experiments were repeated several times. The anus of the nymphs was occluded with paraffin and they were then exposed for 4 and 24 hr. at 30° C. in dry air, and the percentage loss of weight noted. A selection from the results is shown in Table 13.

The substances used show a very wide range of activity in increasing the rate of loss of water, but certain general principles can be discerned. Simple water-soluble wetting agents such as Turkey-red oil, 'Agral', 'Perminal', 'Aerosol OT', have very little effect. Where such water-soluble substances have definite detergent action, as with 'Lissapol' or sodium cetyl sulphate, their effect is somewhat greater. Refined petroleum oils (P31) have very little effect, and so have olive oil and lanoline. But when oils with polar groups such as ricinoleic acid, linoleic acid, oleic acid, or the naphthenic acids are applied, the effect is much increased.

In general it is the oil-soluble substances with hydrophilic properties that are most effective. This

Table 13. *Loss of weight in 4th stage nymphs of* Rhodnius, *24 hr. after feeding, exposed at 30° C. in dry air after smearing the dorsal surface of the abdomen with various chemicals*

Chemical substance	Trade name or code number and maker	Loss of weight % in 24 hr.
Control	—	1·5–2·5
Sodium salt of a sulphonated naphthalene derivative	Perminal BX (I.C.I.)	2·4
Sodium salt of a sulphonated aromatic hydrocarbon	Agral 2 (I.C.I.)	2·9
Refined medium petroleum	P31 (white oil) (Shell Co.)	3·3
Ammonium salt of ricinoleic sulphuric acid ester	Turkey-red oil	3·4
Carboxylic acids from petroleum. Acid value: 230mg. KOH/g.	Naphthenic acid NA9 (Technical Products Ltd.)	3·6
B-naphthyl ether of polyethylene glycol, $R(OC_2H_4)_8OH$ (approx.)	R1660 (I.C.I.)	3·75
B-naphthyl ether of polyethylene glycol, $R(OC_2H_4)_{10}OH$ (approx.)	R1661 (I.C.I.)	3·9
'Lecithin'	Alco Soya Lecithin (American Lecithin Co.)	3·9
Sodium secondary alkyl sulphates (chain length C_{10}–C_{18})	Teepol X (Technical Products Ltd.)	4·2
A phthalic glycerol alkyd resin	Emulsifier B1956 (Röhm and Haas)	4·3
Condensation compound of naphthalene sulphonic acid and formaldehyde	Belloid T.D. (Geigy Colour Co.)	4·5
Polyglycerol ester of coconut oil fatty acids (glycerol: fatty acid = 20 : 4)	Lever Bros.	4·5
Ricinoleic acid	—	4·6
Polyethylene glycol monoiso-octyl tolyl ether	Triton NE (Röhm and Haas)	4·6
A straight chain sodium alkyl sulphate	Lissapol C (I.C.I.)	4·9
Diethylaminoethyloleylamide acetate	Sapamine A (Ciba Co. Inc.)	4·9
Complex alkylated benzene ether of polyethylene glycol, $R(OC_2H_4)_8OH$ (approx.)	R2206 (I.C.I.)	5·2
An alkyl naphthalene sulphonic acid	Belloid NW (Geigy Colour Co.)	5·8
Triethanolamine soap	Belloid F (Geigy Colour Co.)	6·2
Sorbitol laurate	Glyco Products Co. Inc.	6·2
Sorbitol oleate	Glyco Products Co. Inc.	6·3
Linoleic acid	—	6·4
Twitchell's base	Technical Products Ltd.	6·6
Oleic acid	—	6·7
Polyglycerol ester of coconut oil fatty acids (glycerol: fatty acid 20 : 2)	Lever Bros.	6·7
Sodium cetyl sulphate	I.C.I.	6·9
Sodium salt of sulphonated ethylmethyloleylamide	Igepon T (General Dyestuff Corp.)	6·9
Coconut oil fatty acids mono-diglyceride (88 : 12)	Lever Bros.	7·2
Sulphonated fatty esters	Calsolene oil HS (I.C.I.)	7·2
Diglycol oleate	Glyco Products Co. Inc.	7·5
Sorbitan monoleate	Span 80 (Atlas Powder Co.)	7·6
Petroleum oil containing sodium naphthasulphonate or similar compounds	Soluble 'cutting' oils (Shell Co.)	8·7
Complex sulphonate	Foamacrex (Rex Campbell Co.)	9·1
Complex sulphonate	Emulsifier SZ (Rex Campbell Co.)	9·6
Polyglycerol ester of coconut oil fatty acids (glycerol: fatty acid = 20 : 6)	Lever Bros.	9·6
Polyglycerol ester of coconut oil fatty acids (glycerol: fatty acid = 20 : 10)	Lever Bros.	10·5
Carboxylic acids from petroleum. Acid value: 170mg. KOH/g.	Naphthenic acid NA17 (Technical Products)	10·6
Ammonium laurate	Ammonium laurate S (Glyco Co. Inc.)	11·1
Sodium naphthasulphonate 50% in P31	Technical Products Ltd.	12·3
Diglycol laurate	Glyco Products Co. Inc.	12·4
Polyglycerol ester of coconut oil fatty acids (glycerol: fatty acid = 20 : 20)	Lever Bros.	13·3
Propylene glycol laurate	Prolaurin (Glyco Co. Inc.)	14·1
Sodium alkyl sulphate (C_{17} with slightly branched chain)	Technical Products Ltd.	15·2
Triethanolamine ester	Belloid F.R. (Geigy Colour Co.)	15·7
Glyceryl monolaurate	Glyceryl laurate S (Glyco Co. Inc.)	16·3
Ovolecithin	British Drug Houses	16·7
Cetyl ether of polyethylene glycol, $C_{16}H_{33}(OC_2H_4)_{17}OH$ (approx.)	R2204 (I.C.I.)	24·7
Cetyl ether of polyethylene glycol, $C_{16}H_{33}(OC_2H_4)_4OH$ (approx.)	R2211 (I.C.I.)	39·5
Cetyl ether of polyethylene glycol, $C_{16}H_{33}(OC_2H_4)_8OH$ (approx.)	C09993 (I.C.I.)	48·0

is well seen in the series of polyglycerol esters of the coconut oil fatty acids, in which the percentage loss of water rises as the ratio of glycerol to fatty acid falls and the oil solubility increases. Thus with a glycerol/fatty acid ratio of 20 : 2 the loss of weight is 6·7%; 20 : 4, 4·5%; 20 : 6, 9·6%; 20 : 10, 10·5%; 20 : 20, 13·3%.

But these properties do not provide a full explanation of the differences observed. Materials such as Twitchell's base or sodium naphthasulphonate are readily soluble in oil and lead to its spontaneous emulsification in contact with water, and yet they are only moderately effective in increasing the loss of water through the cuticle. The polyethylene glycol ethers of ring compounds (R1660, R1661 and Triton NE), all of which are water-soluble, have very little action. The cetyl ether of polyethylene glycol (C09993) is the most effective

Table 14. *Percentage loss of weight in 4th stage nymphs of* Rhodnius 1 *day after feeding, exposed at* 30° C. *in dry air for* 24 *hr. after smearing the dorsal surface of the abdomen with cetyl ether of polyethylene glycol* (C09993) *at different concentrations in water*

100%	45·3
50%	23·5
10%	11·9
1%	4·5
Control	1·8

It appears therefore that some other properties are involved besides an affinity for both oil and water. It may be that the long cetyl chain of the compounds C09993, R2211, etc., is particularly effective in associating with the long chains of the wax and thus leading to its dispersion.

That there is a relation between the quantity of material applied and the effect produced has been shown by applying C09993, dispersed in distilled water in varying concentrations, as a thin film over the dorsum of the abdomen (Table 14).

It may be that the wide differences between the action of the different materials in Table 13 is determined as much by the cement which covers the wax layer in *Rhodnius* as by the wax itself. If this is so one might expect to find large differences in the relative effectiveness of the materials when applied to different insects—in which differences in the properties of both wax and cement probably occur. A small number of materials showing large differences in their action when applied to *Rhodnius*, have been applied to some other insects and their relative effects compared (Table 15). In all cases the cetyl ethers of polyethylene glycol (R2211 and C09993) were the most efficient substances, but in none was their relative effect so outstanding as in *Rhodnius*. In all these other insects the refined paraffin (P31) was relatively more effective than in *Rhodnius*.

Table 15. *Percentage loss of weight from various insects smeared with different materials* (*see Table* 13) *and exposed for* 24 *hr. in dry air.* Rhodnius *nymphs smeared on the dorsum of the abdomen; the other insects smeared all over;* Rhodnius, Pieris *and* Tenebrio *exposed at* 30° C., Nematus *at* 20° C.

Material	*Rhodnius* nymph	*Pieris* pupa	*Nematus* larva	*Tenebrio* larva
Control	2·0	0·1	7·9	2·5
P31	3·3	4·6	17·7	8·9
Diglycol oleate	7·5	8·6	61·2	16·4
Glyceryl monolaurate	16·3	8·6	54·8	22·9
R2211	39·5	11·6	76·6	29·6
C09993	48·0	11·8	73·8	27·7

Table 16. *Percentage loss of weight from* Calliphora *puparium and pupa during* 4 *hr. at* 30° C. *in dry air*

Object and treatment	Loss of weight %
Normal puparium 24–48 hr. old	1·9
Normal puparium before pupation, thinly smeared with C09993	46·7
Normal puparium after pupation,* thinly smeared with C09993	1·7
Pupa exposed by stripping puparium from posterior half	2·3
Pupa exposed by stripping puparium from posterior half, thinly smeared with C09993	17·2

* As determined by the extrusion of the pupal horns.

of all the compounds (see Pl. 2, fig. 9) and it combines oil solubility with dispersibility in water. But the similar compound (R2204) with the longer polyethylene glycol chain is also a very effective substance although it dissolves readily in water and shows little solubility in oil.

In the puparium of *Calliphora* the effect of C09993, like the effect of abrasive dusts, depends on whether pupation has occurred (Table 16). It is possible, when pupation has taken place, to strip away the puparial shell and smear the exposed pupa with the detergent.

Effect of detergents on the entry of insecticides

It has been shown by Fulton & Howard (1938 1942), Hurst (1940, 1943), Wigglesworth (1942) and others that the solvent used for an insecticide influences greatly its rate of passage through the cuticle. It seems likely that the differences between different emulsifying substances in their ability to break down the waterproof layer of the cuticle will be reflected in differences in facilitating the entry of insecticides. This is a large subject which will require careful study. A few experiments only have been made.

Capsules were secured to the backs of 5th stage nymphs of *Rhodnius* with uniformly thick cuticles, by means of cellulose paint, 1 day after feeding, and left 48 hr. to harden (Wigglesworth, 1942). A suspension of rotenone (Stafford Allen 90%: 0·2 g. in 2 ml. of oil) was then introduced and the capsules closed with cover-glasses sealed down with paraffin. Rotenone in refined paraffin (medicinal paraffin) had produced no effect in 7 days. Rotenone in the cetyl ether of polyethylene glycol (R2211) caused the complete collapse of all the nymphs in 24 hr. Nicotine, 2% in refined paraffin (P31), similarly applied had almost no effect in 2 days; in the cetyl ether of polyethylene glycol (R2211) all were dead in 24 hr.

DISCUSSION

The results described confirm the belief that the layer of the cuticle which is impermeable to water is very superficial and very thin. The effective layer lies in fact over the surface of the epicuticle. The puparium of *Calliphora* forms an apparent exception, for here, as was shown by Hurst (1941), the permeability is not increased if the lipoid layer on the surface is broken down. Hurst attributed this property to the condensed chitin-protein complex of the puparial shell; but it has been shown in the present paper that in the prepupa and young puparium of *Calliphora* the impervious layer is wholly superficial as in other insects and that the hardening of the puparium does not increase its impermeability to water. The greater resistance of the three-day puparium to loss of water is due in fact to the very fragile cuticle of the true pupa. So long as this is unbroken it is far more effective in restricting water loss than the hard puparium that covers it. These observations suggest comparison with other multilayered systems, such as the insect egg (cf. Slifer, 1937).

The waterproof layer is of a waxy nature. Its effectiveness is presumably dependent on the orientation and close packing of the long wax molecules. At a critical temperature, which varies widely in different insects, the packing of the molecules is loosened and water can pass through more readily. As was proved on the cockroach by Ramsay (1935), this will account for the fact that the loss of water by evaporation from insects per unit of saturation deficiency rises with rising temperature—as was found by Koidsumi (1934) in the pupa of *Milionia* and by Evans (1934) in the egg of *Lucilia*. The evidence for these ideas is given in the paper by Beament (1945) on the properties of the isolated waxes.

As shown by Beament (1945) the waxes of the different insects have graded properties; they range from the soft grease with polar characters in the cockroach *Blatta*, to the very hard apolar wax of *Rhodnius* or the pupa of *Pieris*. In the living insect, however, there are further complexities. In the cockroach the greasy layer seems to be freely exposed and to be so mobile that it is largely removed by adsorption on to dusts, and is able to spread slowly back over areas which have been abraded. In other insects the wax is covered and protected to a variable extent by a layer of cement. It will be shown elsewhere (Wigglesworth, 1944*c*) that (at least in *Rhodnius*) this layer is the product of the dermal glands and is poured out over the surface of the orientated wax which is itself the product of the epidermal cells.

The deposition of these layers has not been fully considered in this paper; but the secretion of the long wax molecules by the epidermal cells through the substance of the epicuticle presents an interesting problem. Histologically there appears to be a condensed epicuticular layer of say 1 μ in thickness beyond the limits of the pore canals. According to the estimate of Richards & Anderson (1942) it is unlikely that this is perforated by spaces larger than 0·002 μ in diameter. But the wax molecules are probably something like 0·004 μ in length. The epicuticular pores will not therefore admit the passage of the wax in emulsified form. The wax molecules must either be synthesized *in situ* on the surface of the epicuticle or, associated perhaps with some solubilizing substance, they must pass through the epicuticle singly or in small alined groups. It is conceivable that their discharge in this way may favour the close packing and orientation of the wax molecules needed to form an impermeable film.

This view that impermeability to water is due to a continuous layer of closely packed and orientated wax molecules contrasts with the view of Hurst (1943) who regards the surface of the cuticle (of blowfly larvae) as being not homogeneous but composite and made up of heterogeneous protein/lipoid associations in patches. It is admitted, however, that other materials, often perhaps protein, may be present in the form of a covering layer for the wax. Such materials may even be chemically linked with the wax. In any case it is quite evident that there are great differences in different insects. The permeation of lipoid through the cuticle framework, if it occurs at all, seems to be of no importance so far as the prevention of evaporation is concerned.

The observations described emphasize the living nature of the insect cuticle. The retention of water

seems to be a passive process dependent on the form of the wax layer; but the slightest injury to this layer is reflected in the behaviour of the living cells below. They react as though they had been wounded (Wigglesworth, 1937); they immediately cluster towards the injured site (although the epicuticle has not been visibly damaged) and they then proceed to secrete fresh wax to repair the damage.

The abrasion of this delicate surface layer must be a common occurrence in the life of the insect, and were the insect unable to make good slight injuries to its surface layer it would soon cease to be impermeable to water. In the insect larvae in the soil the abrasion is usually so severe that they have lost their power to retain water. The wireworm, for example, is scratched all over. In spite of its hydrophobe cuticle it loses water by evaporation very rapidly and liquid water passes in and out in accordance with the osmotic equilibrium (Evans, 1943). Thus, although they retain the waterproof cuticle characteristic of their class, the soil insects must remain in moist surroundings.

In discussing the mode of action of fine dusts in causing insects to dry up, Germar (1936) does not altogether rule out the possibility of their sharp edges wounding the cuticle, but he is inclined to doubt this explanation because he could see no injuries under the microscope. The use of ammoniacal silver provides a ready method of demonstrating such injuries to the waxy layer. Mechanical abrasion thus provides a simple explanation of the effect of inert dusts—in the sense that the cuticular surface must be rubbed against the dust if this is to have any action. But, as Alexander *et al.* (1944) and Parkin (1944) have shown, there are very striking differences between different dusts and different insects. Before the true mechanism of their action is understood we need to know more about the physical nature of 'abrasion'. It may be that the attractive forces at the surface of crystalline particles, suggested by Alexander *et al.* as important in the working of the inert dusts, play a part in abrasive action. Parkin (1944) and Alexander *et al.* (1944) have suggested that adsorption of the cuticular lipoid by the dust may be important in interrupting the waterproofing film. This is certainly so in the cockroach in which the 'lipoid' is a soft grease. But in most insects, where the impervious layer is an orientated wax, adsorption is effective only during recovery from abrasion, when new wax is being secreted to repair the interruptions.

The complex nature of the surface layer of the cuticle goes some way to explain the difficulty with which poisons enter by this route. If an insecticide is to pass through the cuticle it must be in a medium which will disrupt both the wax film and its protective layer of cement. As Hurst (1943) says, dispersant action must involve both lipoid and protein components. The possible action of abrasive dusts in facilitating the entry of insecticides will require careful study. It is perhaps the abrasion of the wireworm cuticle which renders it so permeable to arsenic (Woodworth, 1938)—though the normal cuticle of the cockroach (O'Kane & Glover, 1935) and of the tick *Ixodes* (Burtt, 1944) also allow arsenic to pass through.

The importance of different emulsifying agents and detergents in breaking down the protective layers also needs studying from this point of view. Lennox (1940) has pointed out the toxicity of lipoid solvents, which he attributes to the solution of the epicuticular lipoids; and O'Kane, Glover, Blickle & Parker (1940) have noted that wax solvents pass most readily through the cuticle of the cockroach. Klinger (1936) and Umbach (1934) describe the increased permeability of the cuticle after extraction with chloroform or ether (cf. Bredenkamp, 1942 and Eder, 1940) and Hurst (1943) notes that prolonged immersion of blowfly larvae in fat solvents results in an irreversible increase in cuticle permeability to water, alcohol, etc. Lipoid solvents are efficient carriers of insecticides into the insect because of their dispersive action on the packed lipoids (Hurst, 1943; Wigglesworth, 1942). Hurst (1941) has also described more subtle effects: the desiccation of hard-bodied insects when smeared with lecithin; the excessive evaporation from *Tenebrio* larvae when in contact with larvae of *Calliphora*, which is attributed to the more hydrophilic character of the free lipoid in the cuticle of *Calliphora*. Many further examples of this sort of action have been described in the present paper, and the principles involved are dealt with in the paper by Beament (1945) on the isolated waxes.

SUMMARY

Transpiration through the cuticle of insects is restricted by a thin layer of orientated wax on the outer surface of the epicuticle. In some insects at least this wax layer is covered by a thin layer of cement.

When heated to a certain temperature the wax layer shows an abrupt increase in permeability to water. This 'critical temperature' varies widely in different species and in different stages of the same species. It is highest in those insects which are most resistant to desiccation.

In the newly formed puparium of *Calliphora* the impermeable film of wax is wholly superficial as in other insects. But after pupation the main impermeable layer is on the surface of the true pupa. The critical temperature of the pupa is much higher than that of the puparium.

Abrasion of the wax layer results in a great increase in transpiration through the cuticle. Inert dusts cause the desiccation of insects by getting between the moving surfaces of the cuticle and abrading the wax layer. Such dusts in stationary contact with the cuticle will not remove the wax by adsorption; hence they are without action on dead or motionless insects.

(The cockroach is an exception to this. Here the waterproof layer is a soft grease, freely exposed on the surface; it is largely removed by adsorption on to the dusts.)

The places where the wax has been abraded can be demonstrated by immersing the insect in ammoniacal silver solution. The phenol-containing epicuticle stains deep brown only where the protective layer of wax has been removed.

Although the epicuticle shows no visible injury as the result of abrasion, the underlying cells react as though they had been wounded, and growth processes in the epidermis are affected.

Insect larvae from the soil show great but variable evaporation of water. This is the result of abrasion of the cuticle by soil particles. If the wireworm *Agriotes* is allowed to moult out of contact with the soil it has an impermeable cuticle like other insects.

After abrasion the living insect is able to secrete more wax through the substance of the cuticle and so to restore its impermeability. Adsorption of the wax by dusts while it is being secreted interferes with this process of recovery.

Lipoid solvents remove the wax layer from the surface and so increase transpiration. A long series of wetting agents and detergents has been tested. They show widely different effects on permeability of the cuticle to water.

Removal of the wax layer by means of abrasive dusts or suitable detergents increases the rate of entry of insecticides through the cuticle.

I have received valued help in the supply of insect material from Mr S. G. Jary, Dr C. Potter, Mr A. Roebuck, Dr I. Thomas and Dr I. H. H. Yarrow. I am indebted to the Geigy Colour Co., Imperial Chemical Industries Limited, Messrs Lever Bros., Messrs Rex Campbell and Company, and Technical Products Limited for the supply of chemicals and information. Samples of chemicals of American origin were obtained through the good offices of the British Central Scientific Office in Washington. Finally it is a pleasure to acknowledge the large amount of careful technical assistance given by Miss W. Wall.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Surface view of cuticle of *Rhodnius* 5th stage nymph treated with ammoniacal silver. Above: rubbed with alumina; below: normal.

Fig. 2. Section of cuticle of normal *Rhodnius* 5th stage nymph after immersion of the whole insect in ammoniacal silver. No staining.

Fig. 3. Section of cuticle of normal *Rhodnius* 5th stage nymph cut from the fresh insect with the freezing microtome and then immersed in ammoniacal silver. All the epicuticle is stained.

Fig. 4. Section of cuticle of *Rhodnius* 5th stage nymph after rubbing with alumina and immersion of the whole insect in ammoniacal silver. The epicuticle is stained over the crests of the folds.

Fig. 5. Ventral abdominal cuticle of *Rhodnius* 5th stage nymph where it touches the ground. Whole insect immersed in ammoniacal silver after running on fine emery cloth.

Fig. 6. Puparium of *Calliphora*, immersed in ammoniacal silver after rubbing gently on filter paper dusted with alumina.

Fig. 7. Larva of *Tenebrio*, immersed in ammoniacal silver after crawling for some hours on filter paper dusted with alumina. The legs are drawn forwards to show the stained areas where the coxae have rubbed against the thorax. There are abraded and stained areas at some other points, also indicated by arrows.

Fig. 8. Cuticle of 5th stage nymph of *Rhodnius* immersed in ammoniacal silver soon after rubbing with alumina.

Fig. 9. The same, after keeping the insect for 2 days in moist air.

Fig. 10. Cuticle of 5th stage nymph of *Rhodnius* 5 days after rubbing with alumina (the dust being left on), stained with Black Sudan B. The dusted area, which is stained, is above.

Fig. 11. Leg of *Hepialus* larva treated with ammoniacal silver, showing the scratches on the exposed surface.

Fig. 12. Ventral surface of *Agriotes* larva treated with ammoniacal silver, showing abrasion of the ventro-lateral lines of soft cuticle. The scratches in the hard general cuticle are scarcely visible at this magnification (see Pl. 2, figs. 1, 2).

Fig. 13. Side of prothorax of larva of *Aphodius* treated with ammoniacal silver, showing scratches chiefly on the prominent folds.

PLATE 2

Fig. 1. Part of ventral surface of third abdominal segment of wireworm treated with ammoniacal silver. This was a less permeable larva.

Fig. 2. The same, from a highly permeable larva.

Fig. 3. Part of ventral surface of *Pterostichus* larva, untreated, showing sclerites in the soft cuticle.

Fig. 4. The same treated with ammoniacal silver, showing scratches in the sclerites and very extensive abrasion of the soft cuticle.

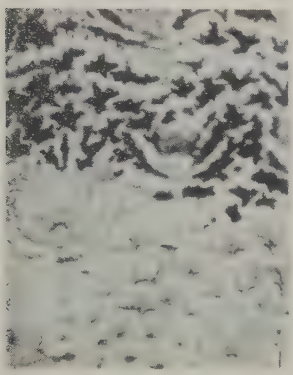
Fig. 5. Cuticle of *Tipula* larva treated with ammoniacal silver.

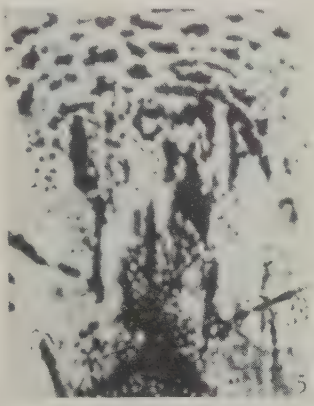
Fig. 6. Cuticle of *Bibio* larva treated with ammoniacal silver.

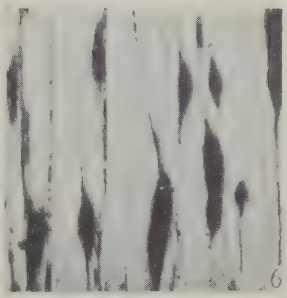
Fig. 7. Cuticle of *Rhodnius* 4th stage nymph treated with ammoniacal silver after extraction with chloroform for 15 min. at 20° C. Staining over the muscle insertion and at small scattered points elsewhere (scarcely visible in the photograph).

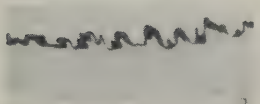
Fig. 8. The same, after extraction for 15 min. at 50° C. Staining almost universal except over the plaques and here and there over the crests of the folds.

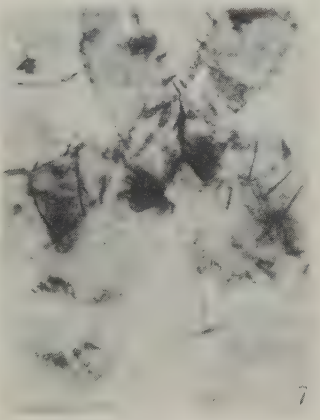
Fig. 9. The same, 24 hr. after smearing with Co9993 (see Table 13). Staining chiefly in the depressions between the folds.

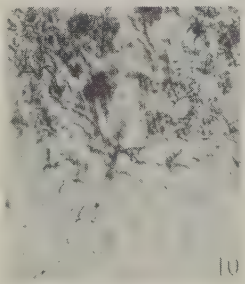


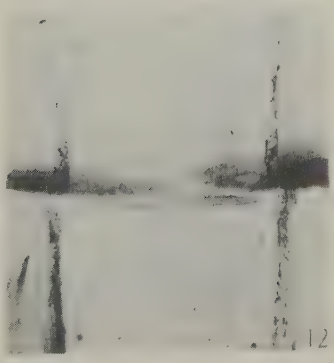




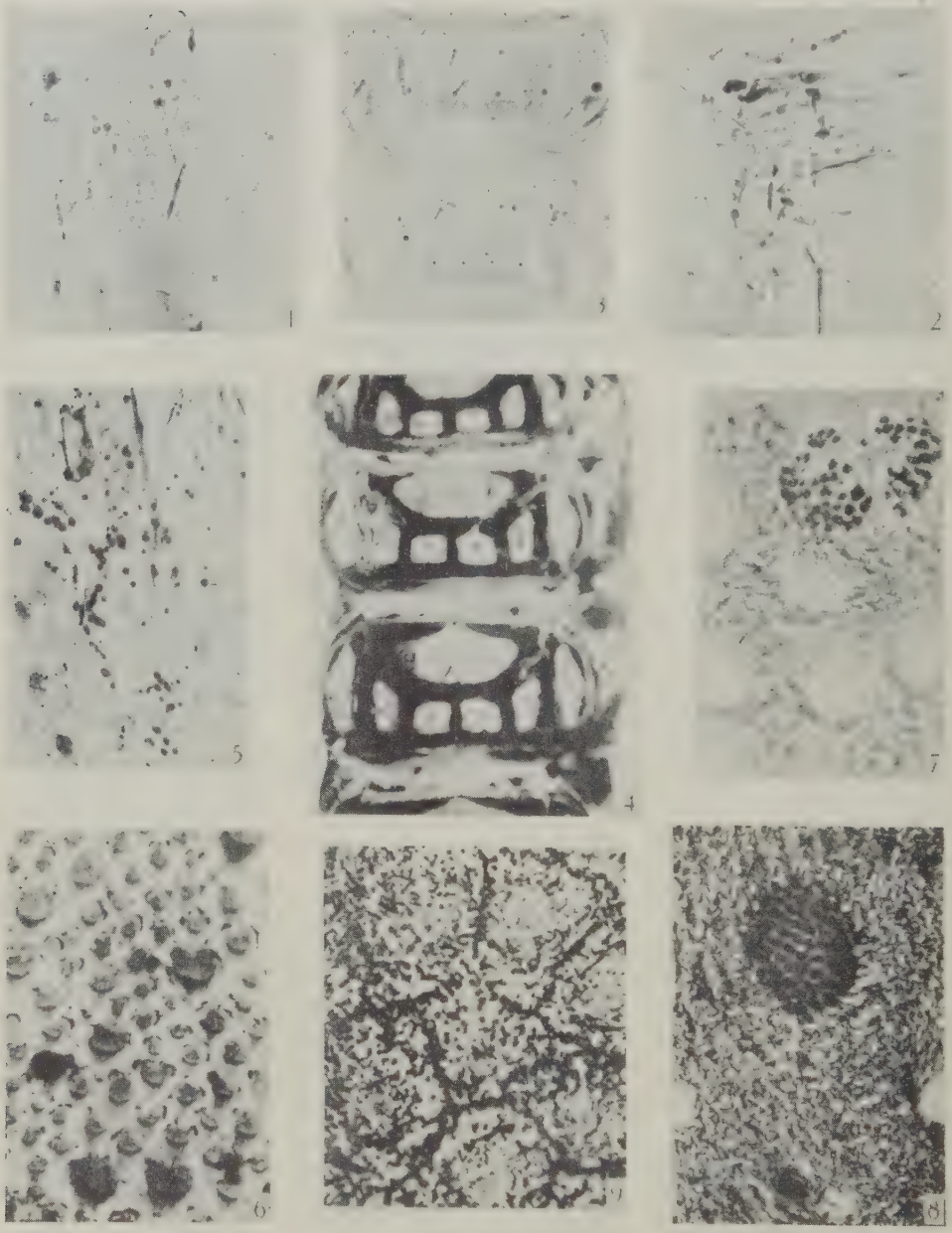








WIGGLESWORTH—TRANSPIRATION THROUGH THE CUTICLE OF INSECTS



WIGGLESWORTH—TRANSPIRATION THROUGH THE CUTICLE OF INSECTS

THE CUTICULAR LIPOIDS OF INSECTS

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(*Received* 14 *November* 1944)

(With Seven Text-figures)

The ability of insects to withstand desiccation was shown by Kühnelt (1928) to be due to the epicuticular layer of the cuticle. This outermost part was found to be highly resistant to strong acids and was shown, by chemical tests, to contain fatty acids and cholesterol type molecules. Wigglesworth (1933) described a complex fatty or waxy substance in the upper layers of the cuticle which he called 'cuticulin'; Pryor (1940) found that this lipoid is entirely confined to the epicuticular layer, suggesting that it is used to impregnate a tanned protein. Lipoid material has also been extracted from the exuviae of the silkworm *Bombyx mori* (Bergmann, 1938). Bergmann found that hot alkali merely fused the epicuticle into oily droplets suggesting that saponification was taking place. The extract so obtained was a brown waxy substance, forming about 4% of the total weight of the cuticle, and it consisted of paraffins, saturated aliphatic acids and esters. A more detailed study of the waxes of certain insects specialising in wax formation has been given by Chibnall, Piper, Pollard, Williams & Sahai (1934*b*); Chibnall, Latner, Williams & Ayre (1934*a*), and Blount, Chibnall & el Mangouri (1937). All these substances were complicated mixtures of alcohols, acids, paraffins and esters with chain length of the order of C_{30}.

Hurst (1941) has suggested that the asymmetry of the insect cuticle is regulated by the 'outer free lipoid'. He postulates a cuticle surface in the form of a lipoprotein mosaic.

It is the object of this study to investigate the distribution and properties of these lipoid materials, to obtain evidence of the structure of the epicuticle by comparison of *in vitro* phenomena with those of living insect cuticle, and to indicate the function of lipoids in resisting the action of certain insecticides.

PERMEABILITY OF WHOLE CUTICLES AND OF EXUVIAE

Apparatus

For direct comparison with the figures obtained for the permeability of living insect cuticle (Wigglesworth, 1945*a*) the standard adopted throughout was the measurement of the rate of passage of water through membranes having distilled water in contact with one side, the other side being exposed to a desiccating atmosphere. The membrane holder (Fig. 1) consisted of a glass tube 1·5 cm. bore and 3 cm. long, drawn out at the upper end to approximately 5 mm. bore and having a short length of rubber tubing attached to this end by celluloid solution. A stout celluloid sheet 4 cm. square, having a central hole 1·2 cm. diameter, was sealed by celluloid solution concentrically to the lower rim of the tube. The whole apparatus was given several coats of thin celluloid solution to ensure waterproof

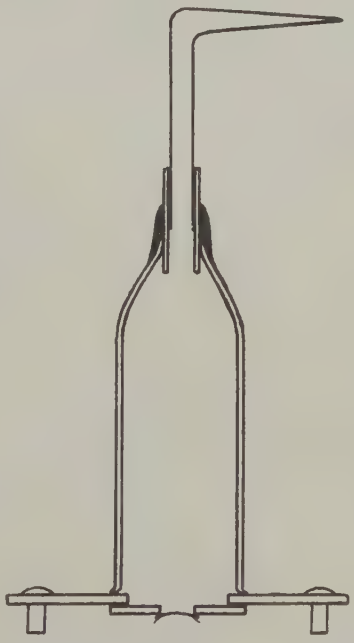

Fig. 1. Longitudinal section of a membrane holder for recording the permeability of whole cuticles of insects.

joints. Three metal studs in the perimeter of the plate supported the apparatus so that the central hole was about 1 cm. from the substrate.

Whole cuticles of *Rhodnius prolixus* were prepared for experiments by dissecting off the upper half of the abdomen of fifth-stage nymphs, 2 days after feeding. Adherent cellular material was carefully wiped out with cotton-wool, and the skin left in distilled water overnight and allowed to dry in the air. A hole 4 mm. in diameter was drilled in a stout celluloid disk 1·5 cm. diameter and the rim coated with cellulose paint. The cuticle was placed on this and the preparation left to dry. A careful examination of the seal and cuticle surface was made under a binocular microscope, and the disk mounted over the central hole of the membrane holder, using celluloid solution as a cement. The holder was then filled with water to a height of 2·5 cm., and a glass tube, drawn into a jet, placed in the rubber tube and sealed in a flame. The whole apparatus was left in a desiccator for 24 hr. before taking readings. The rate of loss of water through the membrane was obtained by weighing at intervals of 24 hr. At the end of each 3 days the sealing capillary was opened to equilibrate any pressure changes, though the amount of water lost was so small as to occupy a negligible volume. Phosphorus pentoxide was used in the desiccator and the membrane, in all experiments, was kept at approximately 5 cm. from the desiccating surface.

A sheet of celluloid, the same size as the cuticle, was sealed into a similar piece of apparatus and used as a control to check the impermeability of the cellulose paint. Pairs of holders were always used together for experiment and control; both had had the same history of exposure to humidities, and the small amount of water absorbed on the surface of the apparatus was the same in both cases. All apparatus was stored in calcium chloride desiccators when not in use.

To compare the permeability of a cast skin with preparations of the above type, the abdomen of a fifth-stage *Rhodnius* nymph, before moulting, was coated with a thin solution of gelatin in water, and allowed to dry. The bug completed its moulting processes but could not cast the abdominal part of the exuvia, which was carefully removed by cutting along the line of the spiracles. The gelatin was dissolved off by washing in several changes of water at 35° C. and the cast skin, free from excreta and undamaged, was dried and mounted as above. By reversing the disk supporting the preparation, the rate of passage of water in either direction can be measured. The exposed area was obtained by camera lucida drawings of the membrane.

Similar experiments were carried out with whole cuticles and cast skins of *Rhodnius* which had been extracted for two half-hour periods with boiling chloroform to remove the waxy substance; and with normal and extracted preparations of the pupal wing case of *Pieris brassicae* and of the wing of the adult *Pieris*. The results are shown in Table 1.

In no case is the permeability of the membrane so obtained as low as that obtained for the living insect (Wigglesworth, 1945*a*). However, the table clearly shows that extraction of the various cuticles vastly increases the rate of passage of water. Whole cuticles of *Rhodnius* prepared as described have, presumably, a number of small perforations, due to the removal of the sacs of the epidermal glands during cleaning with cotton-wool, and the effect of these perforations will be magnified by the pinhole diffusion effect (Brown & Escombe, 1900). The linings of the epidermal glands are left intact in cast skins (Wigglesworth, 1933), and the gelatin coat prevents gross damage due to breaking of bristles in handling. In the wing of the imago there are no gland ducts to be considered, so that this also is a complete membrane.

Table 1. *Rate of permeation of water in mg./sq.cm./hr. through cuticular membranes at* 20° C.

Membrane and treatment	Rate of permeation
Rhodnius, 5th nymph, whole cuticle:	
With epicuticle exposed to desiccator	1·08
With endocuticle exposed to desiccator	1·7
Rhodnius, 5th nymph, whole cuticle:	
Extracted boiling chloroform	12·7
Rhodnius, 5th nymph, cast skin:	
External side exposed to desiccator	0·22
Internal side exposed to desiccator	4·6
External side re-exposed to desiccator	0·8
Rhodnius, 5th nymph, cast skin:	
Extracted boiling chloroform	17·5
Pieris pupa, wing case:	
External side exposed	0·18
Extracted boiling chloroform	5·7
Pieris imago, wing:	
Unextracted	0·29
Extracted boiling chloroform	20 approx.

These results confirm the observation of Hurst (1941) that there is an asymmetry of one hundredfold in the transmission of water through the blowfly larval cuticle. There is a suggestion of asymmetry in the whole cuticle, but in the exuviae of *Rhodnius* the ratio of rates of transmission of water in either direction are 20 : 1 in the initial period, and 5 : 1 after the membrane has been exposed to distilled water for 21 days and is very fragile. It is hoped to deal with the physical processes of this asymmetry in a separate paper (Beament, 1945*b*).

It is clear that the waterproofing layer of the cuticle lies in the exuvia of the nymph and in the pupal shell, which consist of the epicuticle and residues of the exocuticular substance; further, the structure of the epicuticular layer must be asymmetrical and cannot be represented by a regularly impregnated protein layer.

PART I. DISTRIBUTION AND PROPERTIES OF EPICUTICULAR LIPOIDS

Extraction of waterproofing substances

Exuviae of the same stage and similar size were used for the extraction of the waterproofing constituents of the cuticle. *Calliphora* pupal skins were obtained by careful removal of the delicate membrane from the vacated puparium. In each case, about one hundred clean skins were counted, weighed, and extracted with two lots of 10 c.c. of boiling chloroform under a reflux condenser for about 2 hr. The extracted solutions were filtered rapidly through no. 2 filter paper which had been thoroughly washed with hot chloroform. The waxy residues were recrystallized from boiling acetone and weighed. In Table 2 the values given are the average of at least three extractions; the variation in the calculated density was 0·8 g./c.c.; the values do not vary greatly from those obtained by microscopic observation.

It can readily be seen that there is no correlation between the cuticular thickness and the thickness of the waxy layer; in particular, the similar amounts on the very thick *Calliphora* puparium and the very delicate pupal skin are striking. While most of the cuticles have a wax thickness of 0·2–0·3 μ, the much more resistant *Pieris* pupa has a thicker layer, and in *Nematus*, which rapidly succumbs to desiccation, and whose resistance to most insecticides is low, the layer of wax is relatively thin. If we take the average length of a wax molecule as approximately 100 A. (Müller, 1930), and if the film of wax is totally orientated vertically, it represents approximately thirty monolayers.

The unextracted pupal case of *Calliphora* is white and shows interference colours; its ability to take up electrostatic charges makes it difficult to handle.

Table 2. *Thickness of lipoid and non-lipoid material present in the exuviae of various insects*

Species and material	Treatment	Thickness of wax in μ r.d. 0·96	Thickness of exuviae in μ r.d. 0·8	Wax thickness as percentage of exuvial thickness %
Rhodnius prolixus (Hem.), exuviae, 5th nymphs	Washed water	0·25	6·5	3·8
Rhodnius prolixus (Hem.), exuviae, 5th nymphs	Unwashed	0·82 approx.	6·5	12·5
Tenebrio molitor (Col.), large larval exuviae	Washed	0·20	7·6	2·6
Calliphora erythrocephala (Dip.), puparia with pupal skin removed	Washed	0·27	47·0	0·55
Calliphora erythrocephala (Dip.), puparia with pupal skin removed	Unwashed	1·1 approx.	47·0	2·2
Calliphora erythrocephala (Dip.), pupal exuviae	Unwashed	0·18	3·1	5·8
Nematus ribesii (Hym.), exuviae of last larval stage	Unwashed	0·095	6·1	1·65
Pieris brassicae (Lep.), exuviae of last larval stage	Unwashed	0·33	7·3	4·5
Pieris brassicae (Lep.), pupal exuviae	Washed	0·4	16·7	2·4

thickness of a wax layer of a particular example was never greater than 0·05 μ, and the agreement was usually closer. In the case of skins of *Rhodnius* nymphs, *Calliphora* puparia, *Tenebrio* larvae, and *Pieris* pupae, it was necessary to wash the skins in cold water before extraction (see p. 123) in order to get consistent values for the wax thickness. The areas of the skins were determined by camera lucida examination of a number of skins (*Rhodnius*, *Tenebrio*, *Calliphora* pupa); from geometric considerations (*Calliphora* puparia); or from the formula $S = KW^{\frac{2}{3}}$ (S = area (sq.mm.), W = weight (mg.)). The values of K used were: *Nematus* 8·5; *Pieris* larvae 9·8; *Pieris* pupae 8·4; *Rhodnius* 8·1 (Wigglesworth, 1945*a*).

Lewkowitsch & Warburton (1921) give the relative density of insect waxes as 0·96 g./c.c., and this figure was used to obtain the volume of wax and hence its thickness if present as a uniform layer over the cuticle. The thickness of the exuviae was similarly calculated on the rough assumption that their relative

After extraction, the appearance changes to a dull pale brown with no sheen or interference colours, and it does not readily become charged; all these changes are explained by the presence of a waxy film. It is doubtful if interference colours could be eliminated by the extraction of lipoid impregnating a protein. After extraction, some *Calliphora* pupal skins were heated to 140° C. with strong potash for half an hour and subjected to the 'chitosan' test. A complete layer of chitin was present.

Similar changes in the appearance of *Pieris* pupal skins were noted after extraction.

Blattids. The oily waterproofing substance of *Blatta orientalis* (Orth.) (Ramsay, 1935) was obtained by pouring cold chloroform over the abdomen of several animals, and collecting the washings. It was estimated that the thickness of oil was approximately 0·6 μ. Dust used in experiments on the desiccation of the cockroach (kindly supplied by Dr Wigglesworth) was extracted with chloroform, giving further supplies for experimental purposes.

In view of the small yield of waxes, the usual amount obtained from an extraction being a few mg., beeswax was used for the general experiments on permeability, dusts, and detergents. The pure wax was prepared from a sample kindly provided by Prof. Buxton. It was dissolved in boiling chloroform, filtered hot, and recrystallized from boiling acetone.

General properties of the lipoid extracts

With the exception of the 'soft grease' from the cockroach, all the lipoid extracts were solids, increasing in hardness and showing greater crystalline structure as one proceeded to the higher melting-point members.

The waxes from *Nematus* larvae, *Calliphora* puparia and pupae, and *Pieris* larvae were soft and without crystalline form. Those from *Nematus* and *Pieris* were a very pale yellow; the others were white. All were quite readily soluble in cold benzene, chloroform and carbon tetrachloride. *Tenebrio* larval wax, and the wax extracted from water-washed skins of *Rhodnius* nymphs were hard, white and crystalline; they were soluble in cold chloroform though less readily than the soft waxes.

On extraction, the wax from *Pieris* pupae was a deep yellow colour. The yellow fraction was much more soluble in cold chloroform and a reasonable separation was obtained, giving a hard yellow pigment with no crystalline form and making up about 30% of the total extract. The remaining, less soluble fraction was a hard white wax which crystallized in 'hedgehogs'. Without the yellow fraction this would make the thickness of the film on the pupa 0·26 μ, i.e. of the same order as that in the majority.

Table 3. *Approximate melting-points of extracted insect waxes in* ° C.

Wax	Approximate m.p. ° C.
Nematus, larval	36–42, indefinite
Calliphora, puparial	Indefinite
Calliphora, pupal	50–55
Pieris, larval	57 sharp
Tenebrio, larval	57–59
Beeswax	62–64
Rhodnius	60·5 sharp
Pieris (white wax), pupal	67 sharp
Pieris (yellow part), pupal	No change up to 100° C.

Melting-points. Complicated mixtures of waxes do not show sharp melting-points (Piper, Chibnall, Hopkins, Pollard, Smith & Williams, 1931; Piper, Chibnall & Williams, 1934). The approximate melting-points shown in Table 3 were obtained by placing a small filament of wax in a capillary tube, and heating in a flask of medicinal paraffin. An autoclave flask with flat sides was used so that the wax could be observed without optical distortion by a medium-power binocular microscope. On raising the temperature it was found that a visible change took place some degrees below the true melting-point (see Table 8).

Wigglesworth (1945*a*) shows vast increases in the permeability of the cuticle of whole insects at temperatures well below these melting-points. It would appear that some physical changes take place in the wax at this 'critical temperature', and may be associated with the changes that can be seen in the isolated waxes.

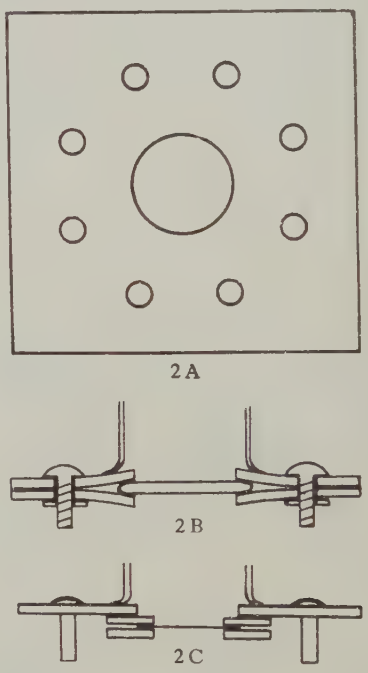

Fig. 2. A. Celluloid plate to support a tanned gelatin membrane in the holder. B. Longitudinal Section of a tanned gelatin membrane in the holder. C. Longitudinal Section of a *Pieris* wing membrane in the holder.

Waterproofing properties of the extracts

Apparatus. To test the waterproofing properties of the waxes, they must be spread on a membrane of known physical properties, and must separate a standard atmosphere from pure water. A modification of the holder shown in Fig. 1 was used. Pairs of identical plates of celluloid (Fig. 2*a*) were prepared, having eight small holes drilled in the perimeter. One of each pair was sealed to the rim of the glass tube and the other could be bolted on to it with eight 4 B.A. nuts and screws (Fig. 2*b*). In

place of the capillary sealing tube at the top, ½ in. lengths of thermometer tubing were used so that expansion of the air could take place with temperature changes, while keeping static diffusion at a minimum.

Membranes. A standardized membrane was required which could be easily sealed into the apparatus for measuring permeabilities. Its permeability should be of the same order as that of insect cuticle after the wax has been extracted, and its surface should be suitable for laying down wax. It must resist attack by water, wax solvents (particularly chloroform) and detergents; and be unaffected by temperatures up to 80° C. The surface should approximate to the chemical type present in the insect epicuticle as this may have an orientational effect on the wax.

No membrane used satisfied all these requirements. Preparations based on paper, parchment, celluloid and porcelain were tried, but the most satisfactory artificial membrane which could be taken up to temperatures of about 60° C. was one made in the following way:

A piece of voile was placed on the bottom of a shallow tin tray and the edges secured by adhesive tape so that it was stretched flat. A hot standard solution of gelatin was poured into the tray, together with a small quantity of benzoquinone solution, so that the thickness was always the same. A number of strips of metal gauze were placed in the hot solution to strengthen the membrane and the tray was heated in a drying oven for about an hour. The soft mass was removed from the tray and allowed to dry. Circular disks were cut from it and held tightly between the two celluloid plates of the holder.

A more satisfactory natural membrane for studying the effect of temperature and of detergents on wax layers was the wing of the cabbage white butterfly, after clearing loose scales by gentle stroking with cotton-wool. All waterproofing layers were removed by two extractions with boiling chloroform for ½ hr. These membranes were sealed between two celluloid rings so that a ½ in. circle was exposed. The completed membrane was fixed over the hole in the membrane holder by cellulose paint (Fig. 2*c*).

The height of water above both types of membrane and in the control apparatus was kept at 2·5 cm., so that the volume of the air space subjected to temperature expansion was directly comparable. The exposed area of *Pieris* wing preparations was measured individually.

Deposition of wax films. A small quantity of the wax was dissolved in chloroform and placed in a pipette, the end of which had been drawn into a very fine jet so that it delivered standard size drops at a slow rate. Fifty drops were run on to a weighed watch-glass and the wax deposit weighed after evaporation. This was repeated and the thickness of wax resulting from the addition of one drop to the area of a membrane was calculated. The wax concentration was adjusted as necessary and the required number of drops added to the cup formed by the membrane and the lip of the celluloid disk. The solution was allowed to evaporate and the resultant layer examined under a binocular microscope for visible flaws. It was found that the rate of evaporation of these solutions was very variable, depending on the particular wax. Therefore, the rates of evaporation of chloroform solutions were tested under comparable conditions by timing the complete evaporation of a standard drop of solution on a glass cover-slip. All readings were taken in the open room during the course of 1 hr. The results are shown in Table 4.

Table 4. *Time for the evaporation of a standard drop of insect wax in chloroform solution*

Wax	Time of evaporation of the drop
Nematus, larval	40 sec.
Calliphora, puparial	45 sec.
Calliphora, pupal	45 sec.
Tenebrio, larval	4 min.
Pieris, larval	11 min.
Beeswax	13 min.
Rhodnius, nymphal	16 min.
Pieris, pupal (white wax)	31 min.

Chloroform solutions of cockroach grease appeared to evaporate quite quickly but the end-point could not be judged.

From Table 4 we see that the higher melting-point waxes have a greater affinity for chloroform molecules, indicating that they are more lipophilic. This is confirmed in Fig. 5. The gradation in properties is remarkably in keeping with the order of the other physical characteristics. It is well known that a slow rate of evaporation of a solvent favours crystal formation in the solute. In the case of waxes from which chloroform evaporated quickly, a small glass capsule was inverted over the membrane cup so that the high concentration of solute vapour slowed the process. The waterproofing powers of such wax films were increased, presumably because the wax layer was more highly organized (see p. 125).

The gelatin membrane had a permeability of approximately 15 mg./sq.cm./hr. when separating distilled water from a dry atmosphere at 20° C.; when coated with wax, the permeability never fell to the value of that of the intact insect from which the wax was obtained.

With the extracted *Pieris* wing, whose permeability was approximately 20 mg./sq.cm./hr., the waxed membrane frequently showed the same order of impermeability as the living insect. It was found with all the waxes, but particularly with the softer ones, that the membrane had become much less permeable after it had been taken past its 'critical

temperature' (see p. 122), and returned to room temperature. This is shown in Table 5.

Table 5. *Permeability of* 1 μ *wax films on* Pieris *wing membranes at* 25° C. *after subjection to the 'critical temperature'*

Values in mg./sq.cm./hr.

Wax	Permeability before C.T.	Permeability after C.T.
Nematus, larval	1·2	0·42
Calliphora, puparial	1·25	0·064
Calliphora, pupal	0·86	0·39
Tenebrio, larval	0·71	0·32
Pieris, larval	0·56	0·15
Beeswax	0·25	0·07
Rhodnius, nymphal	0·29	0·079
Pieris, pupal (white wax)	1·1	0·33
Pieris, pupal (yellow part)	1·6	No change
Blatta, oil	0·42	0·3

This may be due to the flow of the softened wax over small areas of the membrane not previously covered, or to an increase in the organization of the wax molecules in relation to the membrane surface, when they are in a mobile state. The phenomenon was observed with both types of membrane, but particularly on the butterfly wing. It was not necessary to take the wax up to its melting-point to observe the decrease.

The waterproofing oil from Blatta. A small drop of the oil extracted from the abdominal surface of the cockroach was applied on the end of a needle to the centre of a *Pieris* wing membrane. Over a period of 100 hr. the membrane became progressively more waterproof, and the surface shiny as the oil spread out from the centre.

Table 6. *Permeability of a* Pieris *wing membrane at intervals after a small drop of cockroach oil had been placed in the centre*

Diameter of membrane 0·7 cm. Original permeability of membrane 15 mg./sq.cm./hr. Temperature 22° C.

Time of reading hr.	Permeability of membrane
0	15·0
2	10·2
4	4·4
6	3·8
30	2·5
80	1·0
100	0·35
124	0·35

It can be seen from Table 6 that the permeability falls rapidly during the first 6 hr., by which time we suppose that an orientated and perhaps compressed monolayer has been formed over the membrane surface. Slow compacting of the monolayer, or the even distribution of the remaining oil, may account for the time taken for the value to become constant (see Table 16).

The cockroach oil spreads rapidly on water, and it would appear that 6 hr. is rather a long time for a similar spread on a membrane. However, Wigglesworth (1945*a*) shows that after rubbing the tergum of *Blatta* with dust, which removes most of the oil, recovery takes place so slowly that after 48 hr. the insect has not regained its original impermeability.

For membrane experiments it was found that the most useful thickness of wax which could be relied upon to give repeatable waterproofing values and

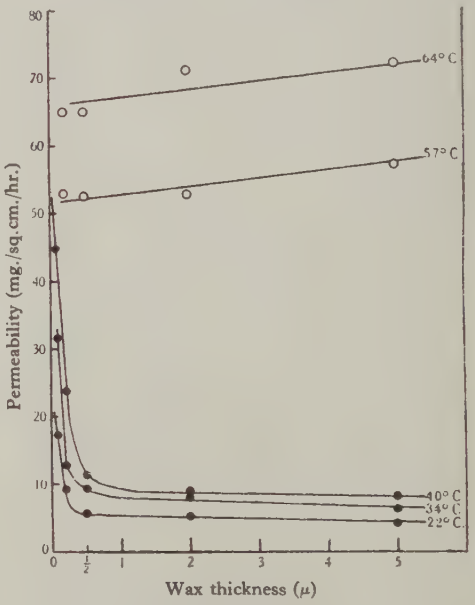

Fig. 3. Graph showing the variation of permeability of a tanned gelatin membrane coated with various thicknesses of beeswax at different temperatures.

stand up to high temperatures was 1 μ. This is vastly in excess of the average thickness on the insect (0·2–0·3 μ). Experiments on tanned gelatin confirmed the observation of Alexander, Kitchener & Briscoe (1944*a*) that the initial layer of wax has the greatest waterproofing effect, and that added thickness does not increase waterproofing regularly. Fig. 3 shows that at low temperatures, a thickness of between 0·2 and 0·3 μ is responsible for most of the impermeability of the wax film. Alexander *et al.* found a similar phenomena on celluloid membranes, occurring at a thickness of 20 mμ.

In artificial systems, the technique of depositing waxes may be such that only with a thickness of wax of 0·2 μ can one be sure of having a wax film

complete over the whole surface of the membrane. The readings are, however, reasonably reproducible and the phenomenon does not occur if *n*-paraffins are used. The orientated molecules at the membrane interface may be able to effect a similar alinement on the overlying molecules, so that, at any rate, the micelles (if not crystallites) of wax are orientated up to this thickness. Above this level, wax, although it may be partially crystalline, is present in random form, and so the effect of increasing its thickness will be small compared with the impermeability of the highly organized lower layers. The latter explanation seems to be the more probable.

Beyond the 'critical temperature', when we suppose that the orientation has broken down, the difference disappears. There is a great deal of other evidence for the presence of an orientated layer of wax at the interface. In the experiments of Alexander *et al.* (1944*a*) the smaller thickness may be due to the lesser degree of orientation induced by celluloid as compared with a tanned protein of the gelatin type; orientation induced by the insect epicuticle may be even greater. It may, therefore, be a point in the insect economy to use the average thickness of 0·25 μ of wax which seems to be the limit of induced orientation; any further increase in its wax layer would not make it vastly more impermeable. Wigglesworth (1945*a*) shows that in recovery from abrasion the waxes are laid down in a much rougher state, and that although the amount of wax present is greater than in the normal insect, the original degree of waterproofing is never recovered.

The effect of temperature on wax film permeability

If the lipoid material in the insect cuticle is present as a uniform layer it should show the same variations in permeability to water at increasing temperatures, whether on the insect or on an isolated system. The waxes were laid down on three types of membrane. Series were obtained with films of 1 μ of wax on each of unglazed porcelain, tanned gelatin and the extracted wing of the cabbage white butterfly.

Experimental procedure. The permeability of the unwaxed membrane was found by weighings after 4-hourly intervals in a desiccator. The wax was laid down from chloroform solution, and the permeability of the waxed membrane taken over periods of 24 hr. at room temperature. An initial permeability/temperature curve was then obtained using 10° intervals of temperature and 4-hourly exposures. From this curve it is possible to detect the region of the critical point although the curve did not show a very sharp break. The membrane was not taken up to the melting-point of the wax but was returned to a desiccator at room temperature and permeabilities taken until they were constant. This membrane was used for the final curve if its permeability was of a reasonably low order (Table 5). For this, 10° temperature intervals were used until the critical region was obtained, when procedure was by much smaller intervals. Readings were taken every 4 hr. until a constant value was obtained for that particular temperature. The fine capillaries at the top of the membrane holder were left open in experimental and control apparatus. Both pieces of apparatus were placed in a large desiccator having a thermometer with its bulb at the centre of the desiccating space. Phosphorus pentoxide was used as the drying agent and the whole kept inside a thermostatically controlled electric oven.

If Fig. 4 is compared with Wigglesworth's (1945*a*, figs. 1, 2 and 3), it can be seen that the changes in transpiration through the isolated lipoid films are almost identical with those given by the corresponding intact insect. The position of the critical temperature is in close agreement in every case; in Table 7 the temperatures at which the permeability reaches 5 mg./sq.cm./hr. are compared with Wigglesworth's figures for intact insects.

We can only conclude that the state of the wax in the cuticle must be very similar to that on the isolated membrane, i.e. as a continuous and discrete layer. Ramsay (1935) showed that the abrupt break at 33° C. in the rate of evaporation from the cockroach is due to a change of state in the oily film from solid to liquid. He did not, however, get any similar effects with various animal fats on membranes.

We have seen that in the experiments on the melting-points of the waxes, quite big visible changes were taking place below the melting-points. The

Table 7. *Temperature in* ° C. *at which the permeability of wax films, 1·0 μ thick on* Pieris *wing membranes reaches 5 mg./sq.cm./hr., compared with similar values for the source insect (Wigglesworth, 1945a)*

	In vitro		*In vivo*
Blatta orientalis, imago	40·0	(*Blattela*)	42·5
Calliphora eryth., puparium	41·5	(before pupation)	41·0
Calliphora eryth., pupa	54·0		57·0
Nematus ribesii, larva	39·3		43·5
Pieris brassicae, larva	46·2		46·5
Pieris brassicae, pupa	66·4		66·5
Tenebrio molitor, larva	57·1		58·5
Rhodnius prolixus, nymph	61·0		64·5
Beeswax	62·1		—

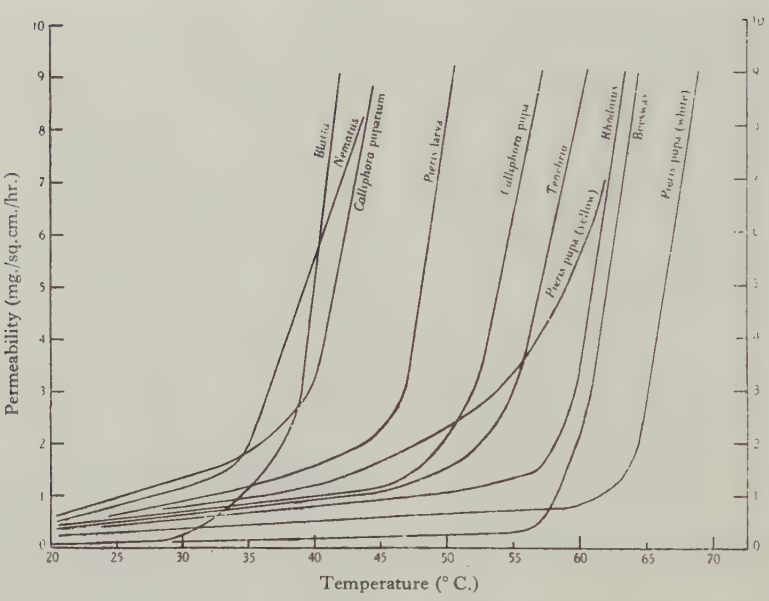

Fig. 4. Graph showing the variation of permeability with temperature of *Pieris* wing membranes covered with approximately 1 μ of wax from various insects.

increase in permeability at the critical temperature may be associated with these changes.

Fig. 4 and Table 5 also show that the waxes with higher melting-points are the more waterproof. Impermeability depends on two factors: the hydrophobic property of the individual wax molecule and the close packing of the whole layer or of a continuous plane of that layer. Fig. 5 shows that the waxes become more hydrophobic, as measured by their contact angle to water, as one goes along the series. Close packing *in vitro* depends on the rate of deposition, on the degree of crystalline structure shown by the wax, and on the changes taking place after heating to the critical temperature.

Physical changes at the critical temperature

A number of physical properties of the waxes were investigated in order to elucidate the changes taking place at the critical temperature in those characteristics relating to affinity for water.

Optical changes. Table 8 gives the changes in appearance of the wax during the melting-point observations. These changes would indicate a crystalline transition at the critical temperature. It can therefore be called a 'transition point'.

Spreading on the surface of water. A large conical funnel was supported in a water-bath so that the rim was about 1 cm. above the level of the water. Cold water could be run in through the bottom of the funnel in order to change its contents rapidly and

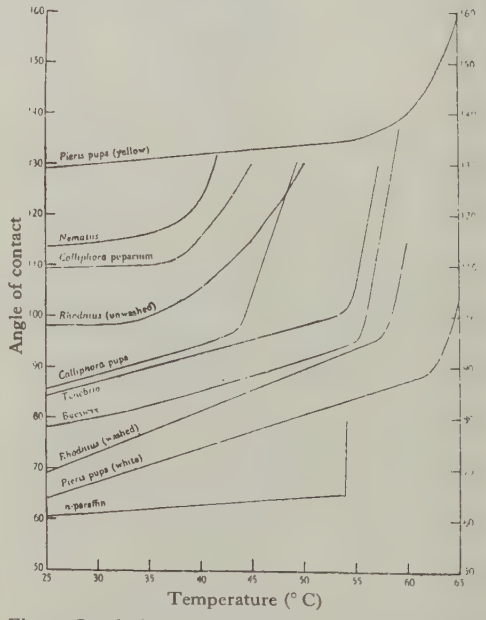

Fig. 5. Graph showing the variation in the contact angle of water against wax films from various insects at different temperatures.

to ensure a clean surface. The funnel was cleaned thoroughly with soap and water, washed through for about ½ hr., and filled with water to within 1 cm. of the rim and a small quantity of lycopodium powder blown evenly over the surface. A number of fine glass rods were drawn and kept in running water to remove all surface active material. Before use each rod was tested for cleanliness by dipping into a dusted water surface. A wax filament was taken up on the end of the rod, and mounted on a vertical worm screw, so that it could be lowered into the surface of the water. This is important, as surface solution occurs only from the edge exposed to the air/water interface and not from immersed crystals (Rideal, 1926). The temperature of the bath was then raised slowly, and a thermometer in the water in the funnel gave a reading probably 1 or 2° above the true temperature of the surface. Similar experiments were carried out on waxes from washed and unwashed skins, on washed and unwashed cuticles, and on the water used for washing. The results are given in Table 9.

In the case of *Pieris* larva and *Calliphora* puparia there is spreading from the washed skin near the transition point. We must presume that in the others the affinity of the lipoid for the cuticle is greater than that for water even above the wax melting-point, or else there is a further layer isolating the wax. *Calliphora* puparia are certainly contaminated with a great deal of polar material (mostly protein and lipoid) from their larval environment, and this accounts for the high figure obtained for the thickness of wax extracted from the unwashed puparia (Table 2). There would appear to be highly polar substances present in the three highest melting-point waxes. They may be partially removed by prolonged immersion in water (see Table 2). Their effect on the spreading of wax solutions on water was tested in the following way:

Parallel experiments were carried out on two sets of equal numbers of the exuviae of *Tenebrio* larvae, *Rhodnius* fifth nymphs, and *Pieris* pupae; one set was thoroughly washed for 24 hr. in running water, the other left. They were extracted in benzene, so that the solutions would float on water. The cold extracts of these cast skins were poured on to the surface of clean water in large petri dishes, and allowed to evaporate. A fine layer of lycopodium powder was blown over the dish to show the extent of the rigid film present and its area was measured. The ratio of the areas of the extract from washed to unwashed skins was:

Tenebrio larvae	1/4
Rhodnius fifth nymphs	1/10

Washing does not seem to remove the 'spreader' in *Pieris* pupal wax, but the benzene extract of three pupae, total area about 10 sq.cm., when poured on to water, gave a rigid film of over 150 sq.cm.

It is apparent from Table 9 that the waxes in general become much more labile at a temperature a few degrees below the melting-point; this temperature corresponds with the crystalline transition temperature.

Table 8. *Temperatures at which crystals of wax change their appearance*

Wax	Temp. °C.	Observation
Nematus, larval	Indef. changes from 30° C.	
Calliphora, puparial	Indef. changes from 24° C.	
Calliphora, pupal	36–46	Increasing transparency
Pieris, larval	46	Darkens
	54	Outline changes
Tenebrio, larval	51–53	Transparency
Beeswax	57–58	Transparency
Rhodnius	56–58	Darkens
Pieris (white wax), pupal	59	Transparency
Pieris (yellow part), pupal	54	Oily appearance

Table 9. *Spreading from various materials at an air/water interface, over temperature range 20–70° C. Room temperature, 20° C.*

Insect	Unwashed skin °C.	Washed skin °C.	Washing water at room temperature	Wax from unwashed skin °C.	Wax from washed skin °C.
Nematus, larva	None	None	None	At 24	At 29
Calliphora, puparium	Slow at 20	At 27	Rapid	At 20	At 26
Calliphora, pupa	None	None	None	At 42	At 43
Pieris, larva	Slow at 20	At 46	Slow	At 20	At 41
Tenebrio, larva	At 20	None	Rapid	At 48	At 55
Beeswax	—	—	—	At 60	—
Rhodnius, nymph	Rapid at 20	None	Rapid	At 51	At 55
Pieris, pupa (white extr.)	Very slow at 20	None	Rapid	—	At 60
Pieris, pupa (yellow extr.)	—	—	—	—	At 52
Blatta (oil)	Spreading at all temperatures				

Water contact angles of wax films

The measurement of the contact angle between wax surfaces and water is one of the most useful physical properties, as it is a function of the molecular attractive force between the wax surface and water at any temperature.

Contact angles were measured by two methods: that of a water droplet on a waxed cover-slip (Beament, 1945*a*), and the method of tilting a waxed plate until the water meniscus on one side is horizontal. The wax films used were approximately 1 μ thick, laid down on clean cover-slips, and left for at least 48 hr. before use. In the case of the droplet method, readings were taken on either side of the

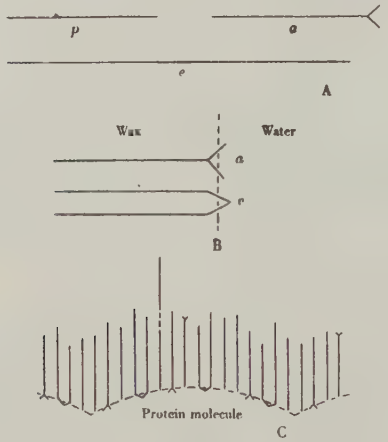

Fig. 6. A. Representation of the main components of insect waxes; p = paraffin, a = long-chain acid, e = ester. B. Diagrammatic representation of the probable orientation of wax acids and esters at a water interface above the transition temperature. C. Diagrammatic representation of the type of orientated monolayer which may be linked to a protein molecule in the insect epicuticle.

drop, and in both cases the angle was measured at every 5° of temperature over the first part of the curve, and at every degree in the region of the transition point. The change in the contact angle is shown graphically in Fig. 5.

The figure also shows the variation with temperature of the water contact angle of a film of a pure *n*-paraffin having a melting-point at 54° C. There is only a very small and regular increase in the angle up to the temperature at which the wax melts, when the water runs up between the wax and the supporting glass. In all the other waxes there is an increase in the hydrophilic properties at the transition temperature and well below the melting-point of the wax. The almost perfect gradation in properties is also seen, for the lowest melting-point waxes have the lowest contact angle, and the highest waxes are the most hydrophobic. *Rhodnius* wax extracted from unwashed skins is much more easily wetted; the transition change is much less abrupt and takes place at a lower temperature. The yellow fraction of the *Pieris* pupal extract is much more hydrophilic than the range of waxes. We thus have further evidence of the presence of a lipoid substance in the higher melting-point waxes which is much less hydrophobic.

Chibnall (1944) states that the insect waxes, having melting points in the range 40–70° C., consist of acids, esters and paraffins. The linking group at the centre of the ester is the most hydrophilic part of the molecule, while the active end of the long-chain acid is much more hydrophilic than the rest of the paraffin chain. At the transition temperature the molecules may become sufficiently mobile to orientate themselves at a water interface with their more hydrophilic groupings outwards, and such changes will increase the adhesive force of water and increase the contact angle (see Fig. 6).

Table 10. *Contact angle of* Rhodnius *wax films against water at room temperature, after various temperature treatments*

Treatment of film	Contact angle at room temperature
Laid down from chloroform	72°
Heated to 51° C.	76°
Heated to 58° C.	102°
After 24 hr.	99°

In order to gain further evidence of this, a film of *Rhodnius* wax extracted from washed skins was used. The contact angle at room temperature was taken, the film immersed in water at 51° C. for 15 min. (below the transition temperature) and the contact angle measured at room temperature. The procedure was repeated, using water at 58° C. (between transition and melting-points). The film was placed in a desiccator and the reading repeated 24 hr. later. The results are shown in Table 10. It can be seen that some permanent change has taken place in the surface wax molecules when exposed to water at the transition temperature, and that on returning to room temperature they retain a more hydrophilic orientation. This cannot, of course, happen to *n*-paraffin chains.

The various temperatures at which physical changes characteristic of the transition point take place have been brought together in Table 11.

Nature of the transition point and structure of the wax layer

Müller (1932) states that with higher *n*-paraffins, having a chain length above C_{24}, the wax molecules show crystalline changes at temperatures a few

degrees below their melting-points, and tend to be packed in a regular hexagonal system. For example, with C_{24} chains, the side spacings are 4·12 and 3·71 A. below the transition point which occurs at 43·9° C., and both values approximate to 4·10 A. at that temperature. The melting-point of the wax is 49·3° C. when the spacings suddenly increase to 4·6 A. These changes at the transition point would cause an increase in the intermolecular spaces. He suggests also that at such temperatures the amplitude of oscillation of the molecule about its long axis may amount to complete rotation. The van der Waal force between the chains would then, presumably, be partially overcome, and the wax molecules become very mobile, while yet remaining in the solid state.

Optical changes (Table 8), permeability changes (Table 5 and Fig. 4), and the variation in contact angle (Fig. 5 and Table 10), all point to similar large movements in the insect waxes at their transition points. The mobility associated with crystal changes would allow orientations of wax molecules in the presence of a suitable surface. If the intermolecular pore spaces increase, the rate of passage of water through them will also rise.

On a grosser scale, changes in the crystalline form of a homogeneous layer of wax will leave small spaces between the newly formed crystallites on first exposure to the transition temperature. We have seen that in the presence of water (Table 10) wax molecules, in a mobile state, will become permanently orientated with their hydrophilic parts exposed, and so these larger channels will have more hydrophilic linings.

The orientation of lipoid molecules at a protein surface has been postulated by Danielli (1938) and Danielli & Davson (1943) in the structure of the membrane surrounding the erythrocyte. During the present work it has been found that the degree of waterproofing conferred by similar thicknesses of wax was greatest when laid down on a membrane most chemically akin to the insect epicuticle, namely, the *Pieris* wing membrane. Arranging the other membranes in a rough order of permeability when supporting comparable wax films: tanned gelatin, parchment, celluloid, porcelain, we see that as we descend the series, membranes are successively less able to present a surface chemically akin to the tanned protein of the epicuticle.

A preliminary X-ray investigation of waxes from these insects has suggested that when they are supported on a fine-silk meshwork, the general bulk of the wax has an orientation, probably in the form of groups of aligned micelles; but these are of random distribution. On the other hand, the wax molecules around the silk fibres appear to be in highly orientated crystallites, and this may be due to the organization of their initial layer by the silk protein.

Table 11. *Comparison of the transition point temperatures in* ° C. *obtained from various physical characteristics*

Insect	Optical changes	Spreading	Contact angle	Permeability of film		
				On tanned gelatin membranes	On *Pieris* wing membranes	*In vivo**
Nematus, larva	30	29	37	32	34	35
Calliphora, puparium	—	26	36	29	37	35
Calliphora, pupal	36–46	43	44	42	49	52
Pieris, larval	46–54	41	42	46	46	42
Tenebrio, larva	51–53	55	53	—	53	52
Beeswax	57–58	60	56	56	57	—
Rhodnius	56–58	55	58	57	57	57·5
Pieris, pupa (white)	59	60	62	—	63	60
Pieris, pupa (yellow)	54	52	—	—	—	—

* *In vivo* experiments (Wigglesworth, 1945*a*).

When the waxes are laid down from chloroform on to the membrane, the film will be crystalline, probably in the form indicated by X-ray analysis for the bulk of the wax. The persistence of chloroform molecules around the higher melting-point members (Table 4) will preclude interfacial orientation, since the hydrophobic part of the molecule is active in both processes. But in all the waxes investigated (Table 5) the lipoid layer is much more waterproof after subjection to the transition temperature, when the molecules are sufficiently mobile to orientate themselves to the tanned protein surface.

The initial layer of an insect wax is the most waterproof (Alexander *et al.* 1944*a*, and see Fig. 3). It is known that orientated monolayers on a water surface can suppress the rate of evaporation considerably (Sebba & Briscoe, 1940), and we can conclude that the extreme impermeability of the lipoid layer of insects is due essentially to an orientated and compact monolayer bound to the tanned protein of the epicuticle (Fig. 6*c*). The

additional effect of the remainder of the wax (0·25 μ) will depend on its close packing, but as most insect waxes contain molecules with chain lengths varying between C_{24} and C_{70}, the greatest organization we can expect is a general alinement, perhaps dependent on the degree of orientation in the initial layer.

The effect of solvent vapour

Insects subjected to chloroform vapour become less waterproof (Wigglesworth, 1945*a*). Competition for the wax, between solvent molecules and the substrate, disorganizes the orientated layer, and the resultant increase in permeability can be demonstrated in two ways.

An unextracted *Pieris* wing had a permeability of 0·23 mg./sq.cm./hr. After exposure for 1 hr. to concentrated chloroform vapour at room temperature this figure had risen permanently to 0·68 mg./sq.cm./hr. The *Pieris* wing is waxed on both sides, so that the inner layer will be affected only by chloroform which has migrated through the wing substance. In view of this, the increase is large and can only be accounted for by the disturbance of the orientation of the sytem by solvent molecules.

Table 6 suggests the formation of a compact orientated layer of cockroach oil on a butterfly membrane after 6 hr., while Table 16 shows that an inert dust cannot remove this monolayer. The values are compared in Table 12 with results of experiments by Wigglesworth (1945*a*) on the dead but intact cockroach.

Table 12. *Comparison of the effect of dust and chloroform vapour during* in vitro *and* in vivo *experiments on cockroach oil*

	mg./sq.cm./hr.
Steady value of membrane permeability with *Blatta* oil after 120 hr.	0·35
Value after 6 hr. spread from a drop in centre of membrane	2·9
Value after dusting (84 hr.)	2·9
	mg./sq.cm./hr.
From Wigglesworth, intact cockroach, approx. perm.	0·37
After dusting	2·5
After exposure to $CHCl_3$	3·1

The effect of chloroform vapour on the whole layer of oil has made the permeability greater than that of the monolayer left after dust adsorption. The only explanation is that the solvent molecules have disrupted the monolayer.

It is important, therefore, that wax solvents should never be used to kill insects if they are to be used for experiments on the waterproofing properties of the cuticle.

Mechanism of orientation. A protein molecule in the epicuticle may accommodate various members of the diverse mixture of acids, esters and paraffins in the wax, at different points on its surface. A diagrammatic representation of the orientated layer is given in Fig. 6*c*. Owing to the dimensions of the intermolecular spaces of the epicuticle, the wax molecules must traverse them in small micelles (unless they are made up from shorter components on the surface), and the first step in orientation will be complete. But most of the orientation will be carried out under the influence of the substrate and, since the pure waxes are solid, some form of solubilizer or emulsifier must be present. The highly polar substances present in the exuviae of insects with higher melting-point waxes may be residues of these emulsifiers (Table 4, Fig. 5, pp. 123, 124 and Table 2). A preliminary investigation has shown them to be soluble in chloroform and other good lipoid solvents; their melting-points are above 100° C. and they have high spreading powers on water. Those from *Tenebrio* larvae and from *Rhodnius* are precipitated as a minute white flocculation when acetone is added to a chloroform solution of the extracts. These properties would suggest that the substances are phospholipids or lipoproteins; they do not respond to chemical tests for cholesterol.

If cast skins of *Rhodnius* are washed with cold distilled water, and the aqueous extracts shaken with a few drops of chloroform, the substances present separate out along the interface between the two solvents and, if left overnight, form a very thin rigid membrane. This aqueous extract will, of course, contain some excretary products. The presence of these substances in *Rhodnius* wax increases the contact angle to water and generally makes the extract more hydrophilic.

The yellow fraction of *Pieris* pupal wax differs in that it is not precipitated from lipoid solvents by acetone; it is present in much larger quantities in the cuticle. Its contact angle, while smaller than that of the whole range of waxes, increases in a similar way with temperature and follows the same type of curve as its permeability changes. It spreads on water at 52° C. and shows a slight change of appearance at 54° C. This substance may be a lipochrome. On the other hand, the high melting-points and more hydrophilic properties may indicate that these small residues are akin to the keto-containing waxes shown by Chibnall *et al.* (1934*a*) to form the main bulk of the extract from *Coccus cacti*, and by Blount *et al.* (1937) from the white pine *Chermes*. In both these insects the wax is present as a filamentous coat which would be useless for waterproofing, and may be an excretary product (Chibnall, 1944) or perform a protective function. There may well be a layer of the beeswax type of wax below the keto waxes, and responsible for the waterproofing. Lipoid extraction takes out all the fat-soluble material in the cuticle, and small quantities of the simpler waxes were present in the extracts obtained from these two insects.

We can conclude that the main barrier to the

passage of water and water-soluble substances through the insect cuticle is a highly organized wax layer some 0·25 μ thick, overlying the tanned protein of the epicuticle, as a continuous layer. This wax layer will be readily penetrated by oil and oil-soluble material, which will upset the orientation of the lowest monolayer, thus bringing about the irreversible changes in permeability reported by Hurst (1943).

PART II. ACTION OF INERT DUSTS

It is well known that certain inert dusts cause desiccation of an insect when in contact with the cuticle (Zacher & Kunicke, 1931; Chiu, 1939; Alexander *et al.* 1944*b*; Wigglesworth, 1944). From experiments on waxed celluloid membranes, Alexander *et al.* (1944*a*) suggest that 'the epicuticular fat film may be preferentially attracted by the crystalline forces at the surface of solid particles, and adhere and orientate itself on the crystal rather than on the relatively structureless cuticle surface. These may even be spread over the particles by surface migration.' We have seen above, however, that the cuticle surface is part of a highly organized physicochemical system binding down the wax molecules, and that insect waxes are mobile only at temperatures above the transition point. Wigglesworth, on the other hand, attributes the effect of these dusts to abrasion. While in the limiting case these two actions depend on the same physical processes, the experiments below were carried out to obtain additional evidence.

Method

Insect waxes were laid down on various membranes, and the steady value of the permeability was recorded. Dust (from the sample of alumina used by Wigglesworth, 1945*a*) was sprinkled thickly on to the membrane surface with the holder inverted, and left in this position for 15 min. Loose dust was gently shaken off and the apparatus returned to the desiccator. In a series of experiments such as the effect of dust over a temperature range, the membrane was redusted before each new temperature.

The presence of dust on wax films on the extracted wing of the butterfly has no effect at any temperature on the rate of transpiration through either the hard wax from *Rhodnius*, or through beeswax, over the range of thickness 0·25–1·0 μ.

Experiments on the soft wax of the *Calliphora* puparia suggested a very slight increase in permeability at room temperature, but the effect of dust at any temperature was by no means as obvious as that on the live and moving insect. On the other hand, the very slight abrasion brought about by spreading dust on the waxed membrane with a camel-hair brush is sufficient to increase transpiration by 100%. Much more dust adhered to the tanned gelatin membranes, though microscopic observation showed that there was always a fine film on *Pieris* wing preparations. We can see that dust has a considerable effect on a wax film spread on tanned gelatin, and the increases here are comparable with those obtained by Alexander *et al.* (1944*a*) using beeswax films on celluloid. It may well be that on celluloid and tanned gelatin, the wax film before the application of dust is by no means complete and continuous, seeing that the permeability is so much greater than that of the insect, or waxed *Pieris* wing. This would account for the extra amount of dust adhering to the membrane. Attraction between the dust surface and the irregular areas of the film, such as at the edges of small holes, may be sufficient to account for the increase.

It is much more likely, however, that these differences are due to the bonding force between the substrate and the wax molecules. In celluloid and tanned gelatin systems we have seen that the lipoid layer is neither so highly organized nor so strongly attached, and the surface attraction of the dust crystal forces described by Briscoe (1943) may be stronger than the interfacial bonding. It is only when abrasive action augments surface adsorption that

Table 13. *Permeability in mg./sq.cm./hr. of dusted and undusted layers of* Rhodnius *wax* (1·0 μ) *on* Pieris *wing membrane at various temperatures*

° C.	Undusted	Dusted	Redusted
20	0·31	0·34	—
30	0·68	0·8	0·60
50	1·6	2·1	1·8
58	15	12·8	10

Table 14

(*a*) Permeability in mg./sq.cm./hr. of tanned gelatin membrane (original permeability 20 mg./sq.cm./hr.) with 0·25 μ beeswax, after treatment with dust at 25° C., values in mg./sq.cm./hr.

Permeability before dusting	3·5
Add dust: After 2 hr.	6·3
After 4 hr.	7·0
After 24 hr.	10
After 48 hr.	11

(*b*) 0·25 μ beeswax on *Pieris* wing membrane (original permeability 24 mg./sq.cm./hr., 22° C.).

Permeability before dusting	1·1
Permeability after dusting (over 48 hr.)	1·2

Table 15. *Effect of dust sprinkled, or rubbed, on* 0·5 μ *of beeswax on* Pieris *wing membranes at* 20° C., *values in mg./sq.cm./hr.*

A.		B.	
Before dusting	1·45	Before dusting	1·40
After sprinkling	1·50	Rub on dust with camel-hair brush	2·8
		Over next 6 days	3·05

dusts increase transpiration through lipoid films linked to epicuticular substance.

The effect of dust on the oil extracted from the cockroach is in direct contrast (Table 16).

Table 16. *Continuation of experiment on membrane used in Table* 6

Original permeability of *Pieris* wing membrane		15·6 at 25° C.
With thin film of oil from *Blatta* abdomen		0·35
Dust added and returned at once to desiccator:	After 1 hr.	1·2
	After 2 hr.	1·75
	After 17 hr.	2·7
	After 84 hr.	2·9

Recalling Table 6, we find that the dust is able to compete for most of the oil over the membrane surface, and causes a large increase in the rate of passage of water, but that it cannot increase this beyond the value obtained when the oil has spread from a central drop for 6 hr. This value was taken to indicate the formation of a completely orientated and possibly fully compressed monolayer over the membrane surface. Hence, while the dust can remove all the superficial oil, it cannot compete for the layer of extract bound on to the epicuticular surface, even though all evidence points to the extreme mobility of this substance. It is therefore certain that stationary dust cannot remove the solid and non-mobile lipoid of the average insect, bound to the epicuticle (and perhaps protected by a cement layer).

Alexander *et al.* (1944*b*) have correlated the effectiveness of a dust with its relative hardness, and shown that dusts are still effective when coated with a few monolayers of fatty acids, though not when coated with thick films. They have also shown that angles rather than surfaces are the most important consideration next to hardness. This evidence is far more in favour of abrasion than surface action, which is very dependent on the actual molecular surface exposed.

PART III. ACTION OF EMULSIFIERS

The waxed membrane technique was used to make a preliminary investigation of the mode of action of a select series from the emulsifiers and detergents, which cause an increase in the permeability of the insect cuticle by breaking up the wax layer (Wigglesworth, 1945*a*).

An emulsifier depends for its action on having one part of the molecule highly hydrofuge and lipophil, which will attach itself to the wax molecule; the other part, hydrophil, so that when these endings are directed away from the wax molecule they provide hydrophilic channels for the migration of water. It is important, therefore, that the emulsifier itself should not unduly suppress the rate of permeation of water through a membrane. On the other hand, more strongly hydrophilic substances will not prevent the escape of water, but may have insufficient affinity for lipoid material to disperse the wax layer.

Detergents and emulsifiers used		Referred to as
Refined medium petroleum	P 31 (white oil) Shell Co.	P 31
Complex alkylated benzene ether of polyethylene glycol $R(OC_2H_4)_8OH$ (app.)	R 2206 (I.C.I.)	R 2206
Diglycol oleate	Glyco Products Co. Inc.	Diglycol oleate
Glyceryl monolaurate	Glyceryl laurate S (Glyco Co. Inc.)	Glyceryl laurate
Propylene glycol laurate	Prolaurin (Glyco Co. Inc.)	Prolaurin
Cetyl ether of polyethylene glycol $C_{16}H_{33}(OC_2H_4)_4OH$ (app.)	R 2211 (I.C.I.)	R 2211
Cetyl ether of polyethylene glycol $C_{16}H_{33}(OC_2H_4)_8OH$ (app.)	C 09993 (I.C.I.)	C 09993

Small quantities of the emulsifiers were spread on membranes with a fine camel-hair brush, and the film examined for 'completeness'. Since it was found that cellulose paint was sometimes emulsified by the detergent, celluloid solution was used to cement the butterfly wing membrane. The permeability of the detergent film was obtained from 24-hourly readings using calcium chloride desiccators at 20° C.; the results are shown in Table 17.

Table 17. *Suppression of permeability of a membrane by an emulsifier film* (*T.G.* = *tanned gelatin*, *P.W.* = Pieris *wing*), *expressed as percentage of steady value to original permeability* (*at* 20° C.)

		%		
P 31	P.W.	94		
Glyceryl laurate	P.W.	91		
Diglycol oleate	P.W.	16·5		
Prolaurin	T.G.	13		%
R 2211	T.G.	56	P.W.	52
C 09993	T.G.	25	P.W.	16

Comparing this with Wigglesworth (1945*a*, Table 13), it can be seen that R 2211, which is of

the highest efficiency on the insect, is itself comparatively waterproof, while substances as far separated in action as R 2206 and C 09993 have the same order of high permeability. When this suppression effect has been allowed for we have a true measure for the action of the emulsifier on the wax layer.

The effect of the thickness of the emulsifier film was also roughly investigated. In the case of P 31 and the less polar complexes, an increase in thickness caused a proportionate drop in permeability, but thicker layers of C 09993 and R 2211 do not alter the permeability appreciably. There is, for example, only 7% difference between the value recorded for films of R 2211, 0·08 mm. thick and 1·0 mm. Thus to desiccate insects, the thinnest films on non-polar material should be applied; with highly polar substances the amount necessary for complete wax emulsification can be used, regardless of thickness.

Effect of emulsifiers on beeswax films

Membranes of known permeability were coated with standard thicknesses of wax, and the steady values of their permeabilities was taken at room temperature. Films of emulsifiers were applied and the loss of water in successive periods recorded. In the histogram (Fig. 7*a*), the effect of four detergents on beeswax films (0·25 μ) on tanned gelatin is compared with the evaporation from *Rhodnius* when covered with the same substance (Wigglesworth, 1945*a*). Fig. 7*b* shows the effect of a further series on a layer of 3 μ of beeswax on *Pieris* wing membranes. The effectiveness (D) is roughly of the same order as that on the insect (F), though the values are not as widely scattered. The amount by which the pure detergent depresses permeability is shown in column E.

It can be seen that R 2211 would be responsible for the greatest loss of water, were it not for its own impermeability; it is therefore too lipophilic. C 09993 and Prolaurin may emulsify waxes to the same extent, the greater impermeability of Prolaurin accounting for its smaller recorded effect. R 2206 and Diglycol oleate are very inefficient; they are readily permeated by water and are therefore too hydrophilic to have had a vigorous detergent action on the wax. P 31 and Glyceryl laurate are at the other end of the scale for, while they may have completely disorganized the wax layer, they are too lipophilic to allow water to escape.

Though R 2211 is more impermeable, it has a greater effect on the thick wax film than has C 09993. Since it is more lipophilic it can presumably accommodate a greater wax concentration. The effect of wax concentration on C 09993 was then investigated. A thin film of the emulsifier was added to a wing membrane, and the permeability recorded. A chloroform solution of beeswax corresponding to 0·3 μ was added and readings taken to a constant value. This was repeated, so that the amount of wax present was 0·6, 0·9, 2·0, and 6·0 μ by additions to the same membrane. The results are shown in Table 18.

Small concentrations of wax have very little effect on the permeability of the film of emulsifier, but there is a change in the system, with a wax thickness

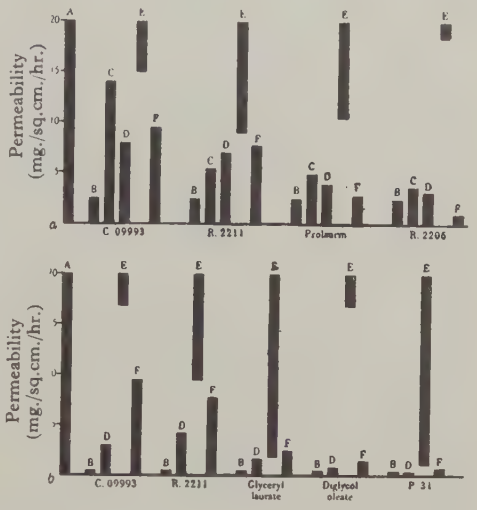

Fig. 7. Histogram showing the permeability of membranes: (*a*) 0·25 μ beeswax on tanned gelatin; (*b*) 3 μ beeswax on *Pieris* wing membranes. A. Permeability of unwaxed membranes. B. Permeability of waxed membranes. C. Permeability 4 hr. after the addition of detergent. D. Steady value of permeability 48 hr. after addition of detergent. E. Depression of the permeability of unwaxed membranes by thin films of the detergent. F. Effect of detergent on *Rhodnius* (Wigglesworth, 1945*a*) reduced to a comparative scale.

Table 18. *Effect of various thicknesses of wax on the permeability of a* Pieris *wing membrane, at* 25° C. *coated with a thin film of* C 09993

	mg./sq.cm./hr.
Permeability of membrane	17·1
,, with thin film of C 09993	15·8
,, + 0·3 μ of beeswax	14·2
,, + 0·6 μ of beeswax	14·0
,, + 1·2 μ of beeswax	11·6
,, + 2·0 μ of beeswax	3·7
,, + 6·0 μ of beeswax	1·9

of between 1 and 2 μ. As the wax has been added in chloroform solution, and the solvent allowed to evaporate off, it cannot be said that these effects are due to any difficulty in removing the wax from the substrate. At the end of the experiment there was an aqueous layer over the membrane surface. This was carefully removed and spread on another

membrane; it had a permeability of approximately 5 mg./sq.cm./hr. The original membrane surface was washed with cold water and then had a permeability of 3·5 mg./sq.cm./hr., so that even in the presence of the emulsifier, some of the wax had been deposited on the membrane, and probably in a partially organized state, as it was rigidly attached. Similarly when C 09993 was added to films of 6 μ of beeswax, the permeability was not markedly increased, but after 48 hr. the upper layer of emulsifier and wax had a permeability of 4·5 mg./sq.cm./hr., while the wax layer left had increased its permeability by some 50%. It was also observed that mixtures of beeswax and C 09993 having a high wax concentration, spread on cover-slips, would deposit plates of wax at the end of 48 hr.; the 'aqueous' solution left above the plates had a permeability of approximately 5 mg./sq.cm./hr.

We can conclude that below certain concentrations, mixtures of beeswax and C 09993 are quite permeable and that the presence of wax does not vastly decrease the permeability of the detergent. Presumably all the wax molecules are surrounded by emulsifying molecules with their lipophilic parts inwards, and hydrophilic groupings outwards. The wax/emulsifier mixture is homogeneous until the amount of wax present is such that a thin layer of the mixture has a permeability of 5 mg./sq.cm./hr. This represents a state when all the lipophilic groupings are used for emulsification, and additional wax is slowly precipitated; this wax may be arranged in a partly organized way. Similar substances, if produced by the insect, could arrange the hard insect waxes in an organized form on the cuticle (see p. 126).

Wigglesworth (1945*a*) shows that the effect of a select series of detergents on the rate of permeation of water through *Rhodnius* cuticle is far more scattered than the effect on other insects. For example, C 09993 and R 2211 are of about the same effectiveness on the *Pieris* pupa, *Tenebrio* larva and *Nematus* larva. The wax of *Rhodnius* is, however, covered by a thin cement (Wigglesworth, 1945*b*), and the detergent has to penetrate this before it can break up the structure of the wax layer. We have seen (p. 126) that chloroform molecules are able to penetrate the substance of an unextracted *Pieris* wing, and to disrupt the wax layer on the opposite side to that exposed to the solvent vapour. Wigglesworth states that the water loss of a *Rhodnius*, weight 140 mg., is approximately 50% when covered over the upper abdomen with C 09993, and left in a desiccating atmosphere for 24 hr. This is equivalent to a rate of loss of 5·8 mg./sq.cm./hr., assuming that an area of approximately 0·5 sq.cm. has been covered by the detergent.

But pure C 09993 is capable of transmitting approximately 21 mg./sq.cm./hr., and is not subject to a big depression in the presence of so small a quantity of wax as that on the *Rhodnius* cuticle. The value 5·8 can therefore only be accounted for by the inability of the detergent to get at all of the wax; and indeed would fit the permeability to be expected if only half of the wax layer was removed.

This is borne out in Wigglesworth's plate 2, Fig. 9, showing the amount of polyphenol staining after the action of C 09993 which has only partially removed the wax.

Similarly, the actual rate of loss from *Pieris* pupae smeared with various detergents is well below the rate to be expected if the wax were fully emulsified (Table 19).

Table 19. Pieris *pupae smeared with various detergents. Rate of loss of water calculated from Wigglesworth* (1945*a*)

Detergent	Approximate rate of loss mg./sq.cm./hr.	Available permeability of detergent mg./sq.cm./hr.
P 31	0·067	1·50
Glycol laurate	0·13	2·2
Diglycol laurate	0·13	21
R 2211	0·185	12
C 09993	0·185	21

It would appear that the effectiveness of the detergent in penetrating the cement layer is as important as its efficiency as a wax emulsifier. Some indication of this is given by experiments in which the detergents were spread on unextracted wing membranes of *Pieris*. Only in the case of C 09993 was there any increase in the rate of passage of water through the membrane. This was of the order of 10%; in all the others, the permeability fell below the starting-point value. Thus the effect of C 09993 is due to its ability to penetrate the wing substance, and disarrange the inner layer of wax as well. This is further support for the idea that the special effectiveness of C 09993 in increasing transpiration is due to its ability to penetrate or disperse the cement layer.

The relative effectiveness of a detergent in increasing transpiration through the insect epicuticle is dependent on:

(1) The amount by which the emulsifier actually disturbs the wax.

(2) The permeability of a layer of the detergent.

(3) The effect of wax molecules on this permeability; in a good emulsifier they will have no effect.

(4) The ability of the detergent to dissolve or penetrate the cement layer, when it is present.

Maximum efficiency may be obtained with a suitable balance of hydrophilic and lipophilic characteristics.

SUMMARY

The passage of water through isolated insect cuticles and exuviae, before and after extraction with lipoid solvents, has been measured. The impermeability of the cuticle is due to a thin continuous layer of lipoid over the epicuticular surface. Transmission of water through unextracted cuticle is more rapid in the direction epicuticle to endocuticle, than when reversed. This asymmetry is most marked in the exuviae.

The lipoid layer is approximately 0·25 μ thick on most of the insects investigated and is independent of cuticular thickness. The thickness of wax on a given species is very constant.

The pure extracted lipoids are solid waxes, except in the blattids, which have a mobile grease. The waxes show a gradation of physical properties corresponding to their melting-points; the hardest are most hydrofuge and are found on the most impermeable insects.

The impermeability of insect wax films, deposited on a membrane, depends on the degree of chemical orientation induced in the lowest layer of molecules by the membrane surface. The monolayer at the epicuticular interface of the insect is completely orientated and tightly packed, and is the main barrier to the passage of water.

Waxed membranes show a sudden increase in permeability at a critical temperature corresponding to that of the insect from which the wax was obtained. The waxes undergo a crystalline transition at this temperature; their molecules become mobile, and the orientated layer is disorganized. In the presence of water, wax surfaces become more hydrophilic at the transition point, and the molecules are permanently orientated in this state.

Boiling solvents must be used to extract waxes from the exuviae; the extracts are all soluble in cold chloroform. The vapour of wax solvents will destroy the orientation of the wax on the cuticle.

Substances having both hydrophilic and hydrophobic properties are present in high melting-point waxes; they may be residues of natural emulsifiers.

Inert dusts increase the transpiration of waxed membranes by adsorbing the lipoid, only if the membrane exerts little orientational force. They cannot overcome the orientational bonds of a wax layer deposited on a membrane of insect epicuticle, unless adsorption is augmented by abrasion. Dusts will adsorb all but the lowest orientated layer of the grease on the coackroach.

Some analysis is given of the mode of action of emulsifiers and detergents, which increase the permeability of waxed membranes, and of the insect cuticle. Their efficiency depends on their own permeability to water, and possibly on their ability to penetrate the cement layer on the insect, as well as on their capacity for emulsifying wax.

This work has been carried out during the tenure of a Research Training Grant from the Agricultural Research Council. I should like to thank Prof. A. C. Chibnall for allowing me to use some of his unpublished work, and Prof. N. K. Adam, Dr J. F. Danielli and Dr A. D. Lees for useful discussion. Finally, I am greatly indebted to Dr V. B. Wigglesworth, who has supplied many of the materials used, for his constant help and encouragement.

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THE BIOLOGY AND BEHAVIOUR OF *PTINUS TECTUS* BOIE. (COLEOPTERA, PTINIDAE), A PEST OF STORED PRODUCTS

VI. CULTURE CONDITIONS

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(*Received* 19 *January* 1945)

(With Eight Text-figures)

I. INTRODUCTION

Ptinus tectus is now a very common pest of warehouses in this country. It is easy to breed in large numbers in the laboratory, and it is therefore a convenient species to use, among others, for biological assay of insecticides. In such tests, it is highly desirable to be able to get repeatable results; this requires the test insects to be of uniform constitution and in a standard condition. Some relevant information about locomotory activity, which may affect mortality, has been provided in previous papers (Bentley, Gunn & Ewer, 1941; Gunn & Hopf, 1942; Gunn & Walshe, 1942; Bentley, 1944); considerable progress has now been made in producing pure lines by brother-sister mating; the present paper is concerned with the culture conditions required to give uniform animals. The work is not complete, but it has to proceed less rapidly for a time owing to the absence of the senior author and it is felt desirable to make the data generally available forthwith and without much discussion of the literature.

II. MATERIAL AND METHODS

The animals used are the descendants of the stocks mentioned by Bentley *et al.* (1941); they have now been kept for several years at about 25° C. in mass cultures. For the past three years they have been bred in tongue jars about 9·5 cm. in diameter and 4·5 cm. deep, as described by Ewer & Ewer (1942). The cultures are now kept in a constant humidity room at 70 % R.H. Each culture is initially populated by thirty adults which are allowed to lay for 7 days on 50 g. of food. These adults are given soaked cotton-wool to drink from; this is removed at the end of the week. Broadly speaking, about 150 adults emerge from such a culture. The pure lines have not been used in the experiments described here, except in one test of variability.

The food used is 'Artox' wholemeal flour with 5 % dried brewers' yeast. This flour is a blend of no. 1 Manitoba and English Red Wheats, freed from weed seeds, ground by old-fashioned millstones, and has nothing added to it or taken from it. We are indebted to Messrs Appleyards Ltd., of Rotherham, for this information. The food is brought into equilibrium with air at 70 % R.H. before use.

The humidity control of the constant-temperature room is a modified copy of that in use at the Biological Field Station at Slough. The sensitive element is human hair; when the humidity falls, a fan blowing over a 10 l. water-bath is switched on and the water is heated electrically. The water level is kept constant by inflow from an adjacent tank with a float valve of the lavatory cistern type. The modification consists of a valve circuit which reduces sparking and the consequent fouling of the mercury (Fig. 1). In experiments, as distinct from cultures, the animals are kept in sealed chambers with a further humidity control by means of sulphuric acid and water mixtures; the reasons for this are given in § III (*c*).

Temperature is controlled by bi-metallic regulators with Sunvic vacuum switches, both in ovens and in constant temperature rooms. Pressure capsule regulation is too sensitive to changes of barometric pressure to be reliable for this purpose. For the experiments at 15° C. a special unit* was constructed. This consists of a 100 l. capacity refrigerator linked by air supply and return pipes to a similar chamber without any refrigerating unit. When the chamber temperature rises, cold air is blown from the refrigerator to the chamber by means of a Goblin vacuum cleaner motor and fan,† this make being chosen because it is exceptional in that the air current is not heated by being passed over the motor. A fan inside the chamber, operated by a motor outside it, automatically switches on when the cooling current is not required, so maintaining

* This unit was paid for by a grant made to Dr Gunn by the Government Grant Committee of the Royal Society.

† We are indebted to Mr C. B. Collins of Goblin Electric Products for the gift of one of these motors in 1940, when they could not be purchased.

a uniform temperature. A low-wattage heater inside the chamber enables constant temperatures very near to and on either side of room temperature to be maintained. The advantage of a thermostat cooled by cold air over one employing a liquid circulation is that the oscillation of temperature inside glass vessels in the chamber is small because of the low thermal capacity of air.

The equilibrium humidity of the food for experiments is adjusted as described by Ewer & Ewer (1942). The weighed small quantities of food used in many of the experiments are made up as follows. A number of spoons were made by knocking a hemispherical depression in a strip of flat brass. From these a set was selected to give the required quantities. In using them, the flour is stirred up to a standard extent, the spoon is dipped into the flour in a standard way and the heap in the spoon is wiped off flat with another strip of brass. The spoon is then emptied into the experimental tube. At first, each lot of flour was then weighed, and those lots which diverged too much were rejected. With practice in the use of the method, however, it became unnecessary to do more than weigh samples. By this method, it is possible to get 230 mg. of flour in each of many tubes fairly quickly with a range of less than ±4 %.

The method of obtaining eggs is described below in the section on oviposition. For the experiments on larval growth, the eggs are freed from flour and put in small glass vessels to hatch. They are examined about the same time each day and all larvae removed, so that all the larvae are known to have hatched during the previous 24 hr.

The end of developmental life is reckoned to be the time at which the adult emerges from the cocoon and arrives on top of the flour. The quantities of flour used were seldom large enough to cause much delay between these two events. A sharper endpoint would have been the date of formation of the pupa or the date of the last moult; but both of these events occur inside a cocoon and often cannot be seen without breaking open the cocoon. The emergence from the cocoon takes place about a week after the last moult at 25° C., and this duration is no doubt complicated by variations in behaviour; but the appearance of the adult on the surface occurs without interference and can always be seen. Consequently the durations of development given below are always from hatching to emergence from the cocoon.

As soon as each adult emerges, it is weighed on a torsion balance (Fabergé, 1938). Its sex is determined by separating the wing covers, squeezing the

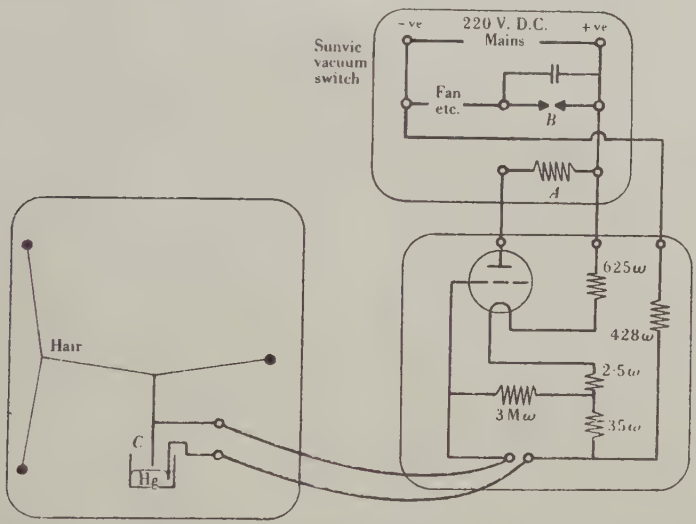

Fig. 1. Humidity control apparatus. The heating element (*A*) of the modified Sunvic vacuum switch is connected in the anode circuit of a triode valve. When the mercury contact (*C*) is opened, the negative potential on the grid of the valve is decreased. This raises the current through the heating element to a value sufficient to close the main contacts (*B*), so that the mains are directly connected to the fan and water heater (see text). The resistances required differ somewhat for individual vacuum switches according to the exact current required to operate the switch; they can be calculated from the characteristics of the valve. The resistance values shown are suitable for a switch requiring 20 mA. and an Osram L.P. 2 valve. We are indebted to Mr A. P. Jarvis of the Electrical Engineering Department for assistance with this circuit.

abdomen slightly, and observing the genitalia which emerge. Hinton (1941) has figured the male genitalia, which consist of a median lobe with a somewhat longer pair of lateral lobes. The female has a telescopic tubular ovipositor with a pair of short setose processes at the end. After examination, the animals are destroyed.

III. AGENCIES AFFECTING DURATION OF DEVELOPMENT AND ADULT BODY WEIGHT

(*a*) *Temperature.* The choice of a temperature at which to keep *Ptinus* cultures—and therefore at which to carry out these experiments—is important. It is desirable to use a temperature at which development is rapid, so as to accelerate results; it is undesirable to use the temperature at which development is most rapid, for the following reason. Temperature-development data are commonly plotted in two ways—speed of development (reciprocal of duration) against temperature, and duration against temperature (e.g. Ewer & Ewer, 1942). The former way gives an approximately straight line, showing that the speed increases uniformly with rising temperature, up to the critical temperature; this line then bends over and begins to go down. The latter way gives a curve which descends to the 'optimal' temperature, where it is horizontal, and then rises for a short distance. The 'optimal' temperature, at which development is quickest, is slightly higher than the critical temperature. At the 'optimal' temperature, the accelerating effect of the last increment of temperature is just balanced by some detrimental effect of that increment, so that there is no resultant acceleration. The so-called optimum is thus a temperature at which some detrimental effect is starting to intrude. It would therefore be unwise to conduct the experiments at the 'optimal' temperature if one wished to draw general conclusions from them.

Ewer & Ewer (1942) concluded that the critical temperature for *P. tectus* lies between 23 and 25° C., while Howe (1943) has quoted 22·5° C. as the optimum. As Ewer & Ewer (1942) pointed out, some uncertainty entered into their results because of difficulties with temperature control, particularly due to enemy action. Accordingly, the durations of larval development and also of complete development from hatching to emergence from the cocoon were determined again at various temperatures between 15 and 25° C. It was found that with ample food (230–250 mg.) and at 70 % R.H., the average durations of larval development and of complete development were some days shorter than those given by Ewer & Ewer (1942) for 15° C., longer for 20° C., and a day or so shorter at 24·7° C. The temperature and speed of development curves plotted from these figures give a critical temperature very close to 24·7° C., i.e. near the upper limit of the range suggested by Ewer & Ewer (1942) (see Table 1 and Fig. 2). Ewer & Ewer worked with animals from cultures bred at room temperature, while our stocks have been kept at 24·7° C. for some years. This may have caused alterations in the stocks which, together with changes in the methods in use here since 1941, might account for the divergences. Thus the temperature of 24·7° C. is not too high for this work, but is the highest which it is proper to use. It is certainly very close to the critical temperature. Perhaps the slightly smaller size reached at this temperature than at 15 and 20° C. (Fig. 3) reflects this fact. In any case, enough experiments have been repeated at 20° C. to show the general validity of the results.

(*b*) *Humidity.* An experiment was carried out to find out what effects slight variations in humidity are likely to have. At 66, 70 and 76 % R.H., the durations of development from hatching to emergence from the cocoon were 54·3, 53·6 and 52·2 days respectively. The differences are in the expected direction but are insignificant for most purposes. Accidental variations of one or two per cent in relative humidity are thus not likely to be a considerable cause of variability in the main experiments. Higher humidities cannot conveniently be used because of the growth of moulds (Snow, Crichton & Wright, 1944).

(*c*) *Quality of food.* These experiments do not pretend to discover the pure substances essential for full and rapid growth (cf. Fraenkel & Blewett, 1943). They are intended to find out what common materials should be used in cultures to produce the best results. The quantity of food required was found in separate experiments (§ III (*d*)) and in the experiments on quality reported in this section an adequate quantity was always present.

Newly hatched larvae were put into separate tubes of 25 mm. diameter with 230–250 mg. of the food being tested. They were kept at 24·7° C. and 70 % R.H. in closed chambers which were opened and ventilated mechanically twice a week. The room in which this was done was kept at the same temperature and humidity, so no disturbance of these conditions occurred when the animals were examined and ventilated. It is advantageous to use closed chambers because this reduces the danger of infestation by mites. Such infestation leads to prolongation of development and to a raised mortality, so experiments with any infested tubes were discarded and ignored in the results given.

Two tests of suitability of the diet were used, namely the duration of development and the weight of the newly emerged adult. The duration of development included larval and pupal duration and also the period from the last moult to the emergence of the adult from the cocoon, which lasts just over a week. Fraenkel & Blewett (1943) did not count this pre-emergence period, and so an adjustment of about 8 days (Ewer & Ewer, 1942) is necessary in comparing their data with ours.

Table 1. *Duration of development of* Ptinus tectus *from hatching to emergence from the cocoon and weight of resulting adults in various diets at* 24·7° C. *and* 70 % R.H. 230 *mg. of food each, one animal per tube*

Diet	Average duration days	Average weight mg.
80 % wholemeal flour + 20 % dried yeast—2 experiments	52·3	3·42
95 % wholemeal flour + 5 % dried yeast—10 experiments	51·2–54·5 Av. 52·9	3·43–3·15 Av. 3·28
94 % wholemeal flour + 5 % dried yeast + 1 % cholesterol	53·0	3·37
N.S.R., casein + 5 % dried yeast	53·8	3·24
99·8 % wholemeal flour + 0·2 % dried yeast	54·0	3·14
99 % wholemeal flour + 1 % dried yeast	54·2	3·07
95 % fishmeal + 5 % dried yeast	54·6	3·23
100 % wholemeal flour	56·4	3·01
100 % fishmeal	58·5	3·28
95 % wholemeal flour + 5 % dried yeast (cocoa stock)	51·7	2·95
95 % 'cocoa' + 5 % dried yeast (lab. stock)	85·9	2·93
100 % 'cocoa' (lab. stock)	123·6	2·24
100 % 'cocoa' (cocoa stock)	133·2	2·05

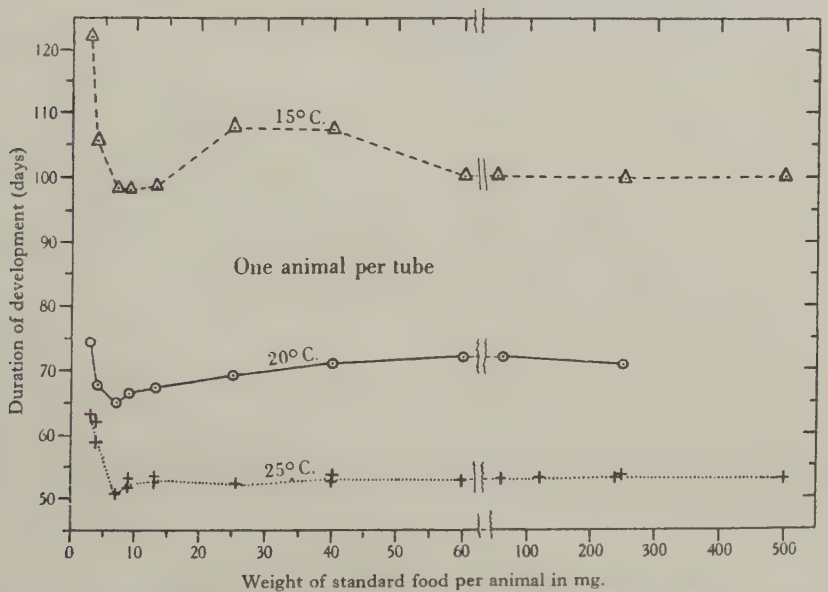

Fig. 2. Durations of development, from hatching to emergence from the cocoon of *Ptinus tectus* allowed various quantities of standard food at 15, 19·7 and 24·7° C. Each point represents the average of at least twenty animals in separate tubes. Note that duration of development is substantially constant unless the quantity of food is very small; development is accelerated at 7 mg. of food and retarded with less. Note repetition of points for 60 mg. of food at the change of scale.

It will be seen from Table 1 that when the results are arranged in order of increasing duration of development, they fall roughly into the order of decreasing weight of adult. That is to say, if the criteria of good food are short development and large adult, then the two criteria lead to the same conclusion about the value of the diet. Wholemeal flour with 5 % dried yeast (now the standard diet) was used as a control for the other diets, and so appears several times in the table. This combination appears to be practically as good as a diet containing more dried yeast and better than one containing more protein and fat (fishmeal). The weight of adults from fishmeal is rather higher than would

be expected from their duration of development. Evidence was obtained from experiments with inadequate quantities of food that a mixture of 50 % Artox : yeast is probably and 100 % dried yeast is certainly less satisfactory than 5–20 % dried yeast and the rest Artox. With 20 animals in an experiment, it is not unusual to get variations of average up to $\pm 1\frac{1}{2}$ days and $\pm 0{\cdot}15$ mg. Consequently variations of this magnitude on the various foods are not necessarily of importance. This is consistent with the results of statistical analysis.

The lower part of the table shows the result of an experiment using a sample of a proprietary brand of cocoa and dried milk which was sent in infested supplement of a salt mixture. The addition of 5 % of Osborne-Mendel salt (Osborne & Mendel, 1913) to 90 % wholemeal flour and 5 % yeast had no beneficial effect on duration. On the other hand, the same supplement to wholemeal flour without yeast was certainly beneficial, and this suggests that the dried yeast supplies whatever salt is necessary and absent from wholemeal flour. On wholemeal flour, perhaps some of the value of the yeast lies in the salt it supplies, rather than entirely on its vitamin content.

(*d*) *Quantity of food.* In these experiments, the standard food (95 % wholemeal flour + 5 % dried brewers' yeast) was used throughout and experi-

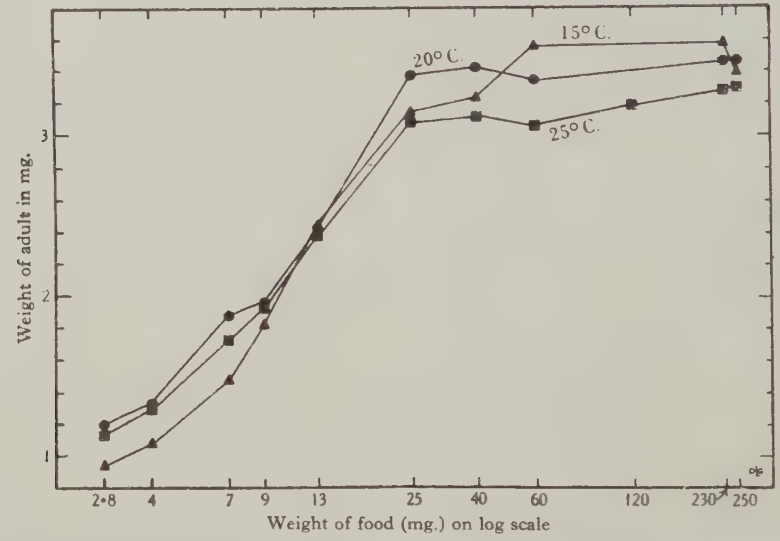

Fig. 3. Weights of freshly emerged adult *Ptinus tectus*, irrespective of sex, when allowed various quantities of standard food, from the same experiments as Fig. 2. About 30 mg. of standard food is required to produce a full-sized adult. The adult weight is not significantly affected by temperature unless the quantity of food is adequate and then the adults are smaller at the highest temperature.

with *P. tectus*. It is at once apparent that this material is not a good diet, even when supplemented by yeast. Nevertheless, the tin contained 331 dead adults, 72 live ones, about 100 pupae and 507 larvae. In the table the experiments marked 'cocoa stock' were done with the progeny of animals from the tin. It will be seen that on standard food they developed as quickly as animals from the laboratory stock, while on the cocoa mixture they developed more slowly. The differences between the stocks were not statistically significant. There is thus no support here for Reynolds's (1943) conclusions on the effect of parental feeding on the duration of development of *Tribolium destructor*.

One experiment was done to find the effect of a

ments were carried out at 15, 19·7 and 24·7° C. The results are shown in Fig. 2. It will be seen that the duration of development was not adversely affected until the quantity of food was very small. Indeed at 7 mg. of food there was a slight acceleration of development at all three temperatures, but with smaller quantities (3 mg.) there was a considerable retardation. Mortality did not usually rise above the common values of 0–10 % until the amount of food was as low as 4 mg. In 2·8 mg. of food at 20 and 25° C., the mortalities were 20 %. At 15° C. one larva died, 11 emerged, and 8 were still larvae 6 weeks after the last emergence; they were discarded. No animals completed development when allowed only 1·3 mg. of food.

An average *Ptinus tectus* brought up in good conditions weighs over 3 mg., yet animals can be reared without high mortality on under 3 mg. of food. They then sometimes weigh as little as 0·8 mg., averaging 1·1 mg. (Fig. 3), and they do not live long as adults. They must make highly efficient use of the food in body building. In fact, on small quantities of food the animals eat it all quite quickly and then feed on the tangled threads of faeces. It is not known whether this has any functional similarity with refection in the rabbit; certainly the faeces contain a high proportion of carbohydrate, even more than the original food.

The weight of the adult is affected by insufficiency of food when there is a comparatively large amount available for the larva. Fig. 3 shows the average

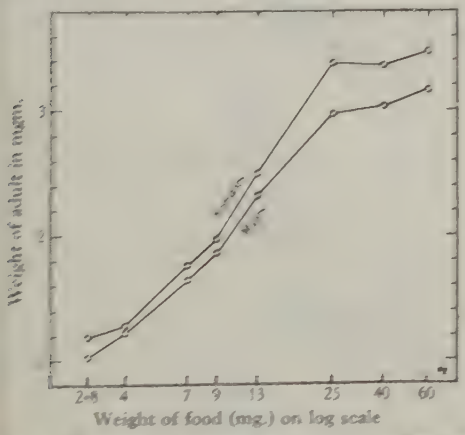

Fig. 4. Weights of freshly emerged male and female adult *Ptinus tectus* from the same experiments as Fig. 2 and 3, irrespective of temperature. There is a difference of sex between the two sexes even when all the food is consumed. The difference is greater when food is available in quantity.

weight plotted against the amount of food on a logarithmic scale. The improvement in size due to increasing the quantity of food is not great above 30 mg. per animal, while 60 mg. of food produces as large an animal as a much greater quantity.

It will be seen that as long as the quantity of food was 30 mg. or more, the lowest average weight reached was higher at 19 and 19·7° C. than the highest average at 24·7° C., but there was no consistent difference between the weights at the two lower temperatures. It is not uncommon for animals growing at lower temperatures to be larger than the same or related species at higher temperatures. For *P. tectus* any such relation seems to be effective only near the optimal temperature.

Below 25 mg. of food per animal, temperature has no clear effect on weight of adult. Plotted logarithmically, all the points fall reasonably well on a straight line which passes near the origin. Such a relation is commonly found with drugs etc. when equal increments of dose produce diminishing increments of effect. Thus while 4 mg. of food produces animals averaging 1·2 mg., in six times as much food the animals weigh less than three times as much.

Some interest attaches to these results from the point of view of the efficiency of body building with limited amounts of food. Generally speaking, newly emerged females are heavier and have longer elytra than males. With ample food at 70 % R.H. and 24·7° C., females average 3·42 mg. and males 3·12 mg. With an inadequate amount of food the differences are disproportionately less. Fig. 4 shows them averaged for the three temperatures used. It is at once clear that with quantities of food which are entirely eaten up, the females always have a greater average weight than the males, and that they therefore somehow use the food more economically in body building. This may mean that they use less of the food for basal metabolism or for locomotion, for there is no difference between the sexes in duration of development. It is noteworthy, however, that Park (1936*a*) found that adult females of *Tribolium confusum* Duval are larger than adult males and that they consume oxygen slightly *faster* than males when motionless, even when the rate of metabolism is calculated per unit weight. It does not follow, of course, that *larval* female *Tribolium* respire faster than males. In the case of *Ptinus* we are concerned with larval metabolism.

The difference of size is greater with larger quantities of food. This may mean that the female eats more when more food is available or it may mean that the male is still less economical—e.g. is still more active—when it can get more food.

As already suggested, the smaller average size of both sexes at 24·7° C. may be due to a detrimental effect of the temperature—if large size is the only criterion, it clearly is detrimental. Here again, the effect may be an indirect one, acting through its influence on behaviour (eating and locomotion). Since larger size is associated with lower temperature only when there is plenty of food, it is likely that larger size is partly due to eating more.

Estimates of the quantity of food eaten by an insect during development have seldom been made. Fraenkel & Blewett (1944) provided food of known water content and dry weight to newly hatched larvae of some stored products insects and then found the water content and dry weight of the residue after pupation. This showed the amounts of dry matter and of water which had disappeared, but of course some of the dry matter at the end was faeces. In *Ptinus* the larva eats its own faeces, especially if short of food, so the amount of food eaten could be found only if the faeces could easily be separated from uneaten food and if food was replaced so frequently and abundantly as to reduce the con-

sumption of faeces to a negligible amount. In our experiments the total amount of food passed into the alimentary canal has not been found; what has been found is the minimal amount of food which must be initially provided to produce an adult of maximal size. We do not know how much of this is eaten more than once.

IV. THE GROUP EFFECT

So far, in §§ III (*a*)–(*d*), we have considered those components of the physical environment which can be fixed in advance. Temperature and humidity remain unaltered throughout the experiment, provided the flour is not deep enough to allow gradients to be set up; but the quantity of food gets steadily less as the animal eats it and the quality alters too, possibly because of selective feeding and certainly because of deposition of faeces. Contamination by faeces might be somewhat relieved by having the bulk of the food small, so that gaseous materials could escape freely, though of course the concentration of solid faeces would be larger. It would therefore be of interest to find the effect of shape of culture—i.e. varying depth with constant weight—on development; but the quantity of food required for a single animal is so small that the standard quantity must be put into very narrow tubes to get any considerable depth, tubes so narrow as to hinder the movements of the animal. Experiments were therefore carried out with several animals together, instead of one animal in a tube. The results suggested that as soon as two or more larvae were put together, some quite new influence was intruding. Accordingly experiments were designed to see whether a group of two animals together in 460 mg. of food behaved differently from two animals each separately in 230 mg. of food. It was soon clear that animals in groups affect each other considerably.

Fig. 5 shows the average weights and average durations of development of groups of *Ptinus* varying in size from 1 to 32. In one set of experiments carried out at both 20 and 25° C., 1280 mg. of standard food were put in each of a number of tubes 2·5 cm. in diameter, with newly hatched larvae varying from 1 to 32 per tube. Thus the quantity of food per animal varied from 1280 to 40 mg. In another set there were 230 mg. of standard food per animal and in another 40 mg. per animal. In another set, tubes of 7 mm. diameter were used with 110 mg. of food per animal. As the graph shows, in all these experiments, when there were several or many animals together in one tube, the duration of development was prolonged and the weight of the adult reduced.

Clearly these effects of breeding the animals in groups instead of singly are not due to a high concentration of animals per gram of food, for they show clearly with 8 animals having 160 mg. of food each (cf. § III (*d*)). They are not due to large bulk of food, for they are conspicuous with 4 animals in a total of 160 mg. of food. They are not due to small surface area of food relative to bulk, for one animal per 7 mm. tube with 110 mg. of food per tube produces substantially normal animals, while 4 animals per 25 mm. tube develop slowly and become undersized, although the surface area is 13 times greater and the depth of food is in one case the same (1280 mg. of food) and in the other case only one-ninth (40 mg./animal) of that in the 7 mm. tubes.

A striking feature of the group effect is that it reaches its highest expression on duration when there are no more than 8 animals in a group. Thus

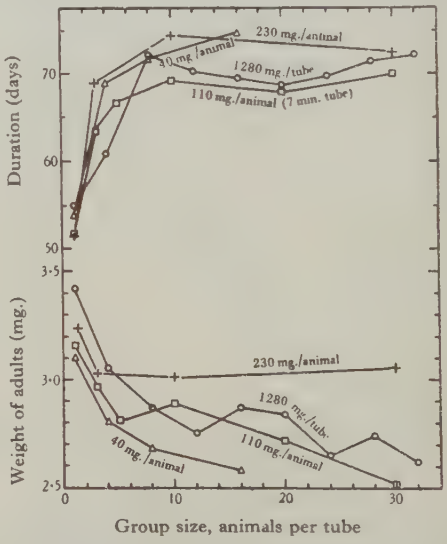

Fig. 5. The group effect on size of adult and on duration from hatching to emergence from the cocoon. With food per animal constant at 40, 110 or 230 mg., the duration of development rises and the weight of the adult falls with rising size of group. There is not much difference between groups of 8 and 32, but a considerable difference between single animals and animals brought up 8 in a tube. The group effect shows just as well with 1280 mg. of food per tube and 1–32 animals in it.

at 24·7° C. the size and duration of development of animals bred singly are 3·3 mg. and 53 days. With animals bred in groups of 8–32, the figures vary somewhat around 2·8 mg. and 71 days, while for animals from groups of 3–5 the values are intermediate. For 20° C., the corresponding figures are 3·5 mg. and 73 days for singles and 2·7 mg. and 109 days for groups. The full group effect is thus an increase in duration of development of say 40 % and a decrease in weight of about 20 %.

The group effect is also found in larger open cultures and not only in the tubes in closed dishes

as described above. Thus in tongue jar cultures open to the air in the room at 25° C. and 70 % R.H., with ample food the average durations of development and the adult weights reached were much the same as in groups in tubes (Fig. 6). It looks therefore, as if the group effect is in some way due to the direct effect of one animal on another. It can hardly be due to 'conditioning' (i.e. contamination) of food, as described for *Tribolium* by Park (1938), for it shows clearly when the quantity of food per larva is constant.

The mortality in group experiments is much higher than with animals brought up singly, but

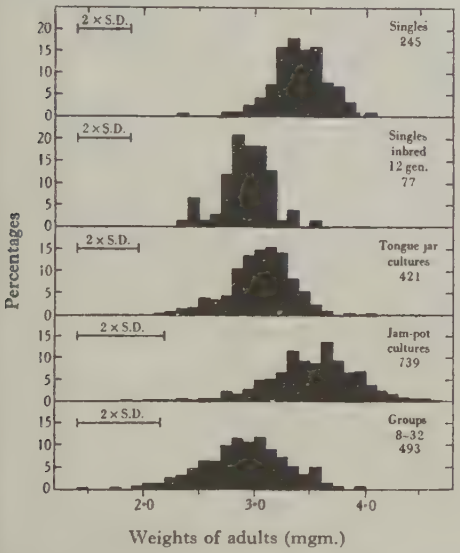

Fig. 6. The group effect on variation in weight of adult. Bimodal distributions have been avoided by adding an appropriate amount (0·3 mg.) to the weights of all males. Note the higher weight, smaller variation and smaller mortality in animals brought up singly. Tongue jar cultures produce results similar to but better than group experiments.

there is no trend with increasing size of group between 8 and 32, such as Park (1938) found in somewhat different experiments. The figures for single larvae are 6 %; for groups of 3–5, 19 %; for groups of 8–32, 33 %; and for tongue jar cultures of 20–200 larvae with 230 mg. of food each mortality was 25 %.

An examination of the data was carried out to find if breeding larvae in groups affects the variability of weight and of duration of development. It was found that the individual durations of development of animals from groups were just as closely distributed about their average as were those of animals bred singly about their average. On the other hand, the variation in weight was much greater for groups than for singles. Fig. 6 shows that the largest animals are almost as big from groups of 8–32 as from single tubes. The size of the smallest animals, however, is much smaller for groups than for singles.

Fig. 6 suggests that in groups there is some component of the environment which reveals the inherent variability of the animals, a component which is absent from the environment of an isolated animal. This hypothesis will be tested using stocks inbred by brother-sister matings; such animals inbred for 12 generations are not less variable than mixed stocks if the comparison is made entirely between individuals brought up singly (Fig. 6).

The group effect is apparent when there is quite a large amount of food, e.g. with only four animals in 1280 mg., and with 10 or 30 animals with 230 mg. of food each. Presumably, however, there must be a concentration of animals so low that the group effect disappears. Accordingly experiments have been carried out with very low concentrations of animals per gram of food and also with very large surface areas of food per animal.

The surface area of the food was varied in one experiment by using 8 g. of standard food and in another two experiments 0·5 g. in containers ranging in diameter from 0·7 to 30 cm. With 8 animals per container, no consistent effect of the consequent variation of area and depth was found. With the largest dishes the animals developed slightly more quickly but they were not heavier; this may have been due to partial virtual isolation of the individual larvae, for the food could not be spread quite evenly and lay in patches.

Varying the quantity of food available did affect the result. Fig. 7 shows the results of all experiments with groups of 8 animals at 25° C. with more than 1 mm. depth of food. It will be seen that the group effect does disappear if enough food is provided, but the quantity required is not less than 5 g. per larva, a hundred times the requirement of an isolated larva. This makes it quite clear that the group effect is in no way due to lack of food, which in any case does not prolong development unless the shortage is severe (4 mg./larva).

It is difficult to see how 'conditioning' or contamination of the food by the animals themselves could have such a striking effect, especially because an isolated animal in 40 mg. of food develops to normal size in normal time. Nevertheless, an experiment was done in which the food was changed weekly, with suitable controls. No significant effect on weight or duration was found.

An experiment was done to see how close the association of larvae needed to be to produce prolongation of development. Partitions of phosphor bronze gauze, of mesh too fine to permit the passage of newly hatched larvae, were put into 2·5 cm. diameter tubes so as to divide them equally into

four. With 230 mg. of flour per animal, 4 animals per tube separated in this way emerged about 4 days sooner than animals not separated but otherwise given identical conditions. Certainly the group effect did not disappear completely or even largely.

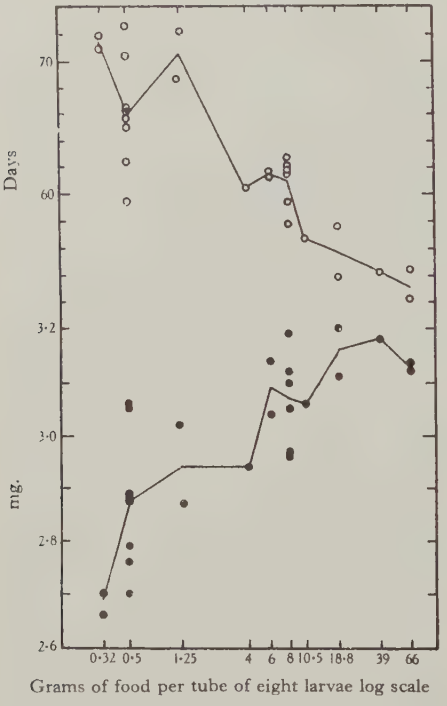

Fig. 7. The group effect with eight animals in various quantities of food. Each point represents two groups of eight animals; the duration of development from hatching to emergence from the cocoon and the weight of the newly emerged adult are plotted against the weight of standard food per tube on a logarithmic scale. Note that even 66 g. of food (8·25 g. per animal) does not produce quite full-sized animals and barely leads to as rapid development as 40 mg. of food for an isolated larva. The concentration of animals must be extremely low before the group effect disappears.

V. OVIPOSITION

The following observations extend those of Ewer & Ewer (1942) and are of some practical importance in the design of culture methods. In all cases, the insects were kept on National Straight Run English biscuit-making flour (N.S.R.) with casein and yeast, with particles small enough to go through a wire sieve having 100 meshes to the inch (say 40 per cm.). No advantage is claimed for this mixture as food over wholemeal flour + 5 % dried yeast, but the former is easier to reduce to suitable fineness. All experiments have been done at 24·7° C. and 70 % R.H. The eggs were collected by gently brushing the flour through a 60-mesh sieve, which retains them. During the sieving, the animals were normally allowed to drink water. It is very important to prevent flour and water coming together on to the animal's body, because a hard cake is formed which frequently blocks the mouth and the posterior openings. Accordingly the animals are freed from flour by shaking in a coarse sieve before dropping them on to sodden cotton-wool. Movements of the mouth parts suggest that they then drink and they do in fact gain in weight. When they have walked off the wet cotton-wool, they are transferred to dry cotton-wool, to remove any droplets on the mouth parts or body surface, and only then are they put back on to flour.

It was stated by Ewer & Ewer (1942) that the female becomes mature between 3 and 12 days after emergence from the cocoon, though insemination may take place within 24 hr. of emergence. Those facts were determined by dissection. The age at which fertile eggs are first laid has now been determined. Females which had emerged during the previous day were mated either with newly emerged males (6 pairs) or with males which had emerged 10–15 days before (7 pairs). They were allowed to drink each day. The two batches gave similar results (Fig. 8 A).

It will be seen that a few fertile eggs were laid within 3 days of mating, i.e. in less than 4 days after emergence from the cocoon. The full rate of laying (5·5 eggs/female/day) was reached on the 7th (5·2) or 8th (5·5) day after mating. The eggs were fertile from the start.

An experiment was also done with 4 pairs in which the males were newly emerged and the females 10–12 days old (Fig. 8 B). These virgin females, which were allowed to drink each day, had been laying 2–4 eggs per day, very irregularly; none of these eggs hatched. After mating, two of them laid at an average rate of only 3 eggs per day, few of which hatched at first; eggs laid after the 17th day showed a good hatching rate. The other two females laid 8 eggs between them in the first 24 hr., none of which hatched, 11 in the second day (2 hatched) and thereafter at an average rate of 5·0 per day, with a normal hatching rate (about 85 %). It is surprising that the fertility of the eggs was low at first, for remated females lay fertile eggs on the first day (see below) while insemination can be performed within 24 hr. of emergence (Ewer & Ewer, 1942).

The rate of laying recorded here and also by Ewer & Ewer (1942) is, of course, far higher than the 10 eggs in a lifetime mentioned by Fahmy (1931). In the first 3 weeks after emergence one female laid 11 eggs on 1 day (with a total of 41 in the surrounding week) while two others laid 10 each on 1 day (57

and 52 in the week). Such high rates are only obtained if the animals are allowed to drink water daily. This is not surprising for two reasons: first, *Ptinus tectus* does not eat well unless allowed to drink (Ewer & Ewer, 1942); second, an egg obviously contains a high percentage of water and weighs about 0·034 mg. (1 % of the weight of the adult). Consequently the deposition of 5 eggs per day must be a serious drain on the water resources of the female. Rough figures indicate that when allowed to drink regularly, a female will lay 5–10 eggs per drink. remating two of the females, 7 eggs were laid in the first day and 5 of these hatched, while the average for the first 5 days was 4·0 eggs per female per day of which 82 % hatched. This experiment shows that fertile eggs can be laid less than 24 hr. after the insemination of the females and leaves open the question of why mature females do not lay fertile eggs for more than 24 hr. after mating with newly emerged males. The slow start made by the two females mentioned above, which were not mated until 10–12 days after emergence, may be

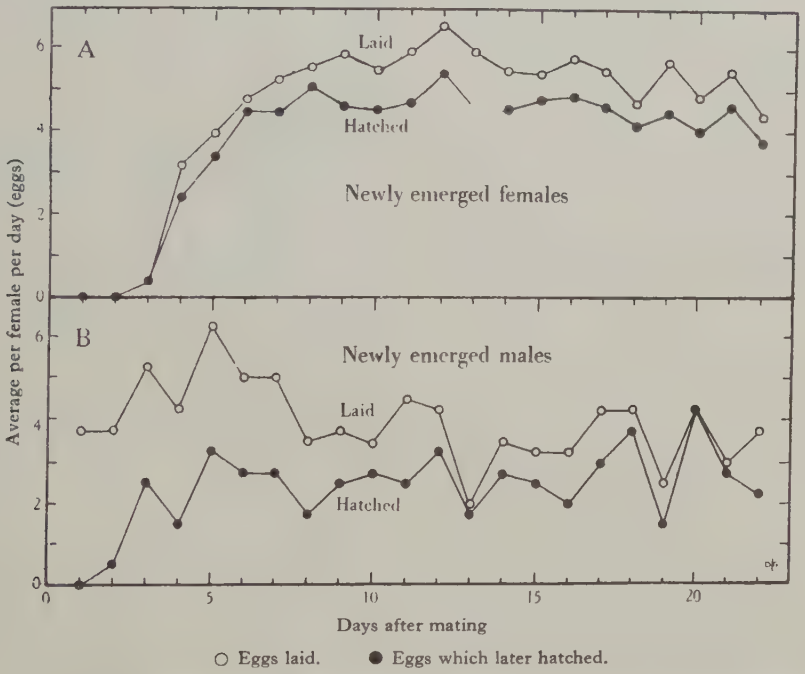

Fig. 8. Average numbers of eggs laid by *Ptinus tectus* and average numbers which hatched later during the first 22 days after mating in pairs. A. both sexes newly emerged from the cocoon (6 pairs) or females only newly emerged (7 pairs). Each female emerged 0–1 day before mating and the first point shows the number of eggs laid in the following day (always zero). B. males newly emerged (4 pairs).

A high rate of laying of fertile eggs occurs 8 days after emergence when the animals are allowed to drink each day.

When allowed to drink daily after weekly drinking, an average of 1·6 eggs were laid on the first day, 2·4 on the second and 5 or 6 daily thereafter. The dependence of a good rate of oviposition on availability of water to drink is now well established.

The influence of the presence of a male on egg laying is considerable. Five females were separated from their mates. They had been laying 5·4 eggs per female per day, of which 83 % hatched. After separation, the rate dropped to 2·8 (73 % hatched) in the first week, 1·1 (51 % hatched) in the second and 1·1 (3 % = one egg hatched) in the third. Upon simply an extreme variant or may be due to some deficiency of behaviour.

Seven of the pairs used in these experiments were kept until one member of the pair died. In the later part of the period they were allowed to drink each third day, when the eggs were collected, except when that day fell on a Sunday; the interval without water was then 2 days. Under these conditions, in 83 days they laid 1124 eggs, a rate of 1·94 eggs per female per day. During this period and during the earlier period under various experimental conditions, each pair produced an average total of 647

eggs (501–803), both members of the pair survived for an average of 276 days (213–370). One female was remated after the death of her mate. She lived 376 days as an adult and laid 960 eggs.

Experiments were done with batches of 30 animals, which were given water to drink on 6 days per week. Five such batches laid an average of 3 eggs per *animal* per day when the food was fresh each day, but the rate fell to 1·6 eggs per *animal* per day on food which had been used for the same purpose several times before, and therefore contained faeces (cf. Park, 1934, 1936 *b*). The sex ratio was not determined exactly because some of the animals died a natural death and the sex is then difficult to discover. The viscera dry up some days before death and the genitalia cannot easily be squeezed out for inspection. There were 62 females and 37 animals of undetermined sex in the total of 150. It is evident, however, that with 30 animals on an area of some 65 sq. cm., the rate of egg laying per *female* is not far below that for isolated couples. The extreme possible values are 4·4 and 7·0 eggs per female per day and the probable value 5–6 eggs per female per day. At this density, crowding seems to have little effect on fecundity (cf. Park, 1933).

VI. CULTURE CONDITIONS

The usual method of breeding *Ptinus tectus* is to put 90 g. of wholemeal flour into a 2 lb. (approx. 1 kg.) jam pot, with a water supply for the adults consisting of a glass tube, 5 cm. long and 2·5 cm. in diameter, three-quarters full of water, with a piece of filter paper projecting from the corked tube. The culture is started by 30 adults 1–3 weeks old, and they are left on for 7 weeks at 25° C. Certain other conditions need not concern us.

We investigated four jam-pot cultures of this type, in which 5 % of dried yeast was mixed with the flour; 739 adults were obtained spread over 6 weeks and individually weighed on the day of emergence. When adults had almost stopped appearing the flour was sieved and 209 larvae were recovered. The distribution of weights of adults is dealt with above (Fig. 6). The average duration of development cannot, of course, be estimated with any accuracy.

By this jam-pot technique, 360 g. of flour produced 739 adults and could have, if more time had been allowed, produced 900–1000. Even allowing for the fact that one culture became heavily infested with Psocids, so that only 123 animals emerged, this means that each animal had about 330–440 mg. of flour, or about ten times the minimum required by animals brought up singly. It has been shown above, however, that the group effect occurs unless a very much larger amount of flour than this is allowed each animal.

Thirty adults are capable of producing at least 3000 fertile eggs in 7 weeks, in good conditions, compared with the average of about 200 adults per culture which actually emerged. There is clearly room for improvement here. No doubt a large part of the deficiency was due to a fall in the rate of egg laying because of the conditioning of the flour by the adults, and there would seem to be no advantage in leaving the parental adults in the culture for so long. Indeed, a decline in the rate of egg laying to half its normal value is shown by animals replaced on used food for a second day or given food used by other animals for 1 day. It therefore appears best to allow a large number of animals in good condition to lay eggs in the intended culture for only 24 hr. They can be brought into good condition by a daily drink and a daily change of flour for 7–10 days, provided they have not been long on contaminated flour.

In the routine tongue jar cultures, in which 30 animals are left on 50 g. of flour, with water to drink, for 7 days, about 150 adults are commonly obtained. By this technique, it is to be expected that the adults will emerge over a much shorter period than they will when adults are left on for 7 weeks.

Some experimental tongue jar cultures were run, in which newly hatched larvae were added to wholemeal flour plus 5 % yeast at the rate of one larva per 230 mg. of flour. There were 6 cultures, two with twenty animals, two with 60 and two with 200. The results did not differ much, so they have been combined. The mortality was 25 %, the duration of development about 70 days, as in the group experiments, and the distribution of weights is shown in Fig. 6.

One test has been carried out with a line inbred by brother-sister mating for 12 consecutive generations. The newly hatched larvae were reared singly in 2·5 cm. diameter tubes, under standard conditions. Their average duration of development was 51 days; it is evident that this inbreeding has led to a reduction in size (Fig. 6).

Fig. 6 is designed to show the variation in weight of newly emerged adults under these various conditions. The graph shows the numbers of animals concerned and the standard deviation. Considering first the animals reared singly, it is at once clear that the inbred animals are small and that, under these conditions, they vary in size just as much as animals not inbred. Had they been brought up in a mass culture a reduction in inherent variability due to inbreeding might have become apparent.

On the other hand, the jam-pot cultures produced animals of large average size but of considerable variety of size. The mean temperature may have been slightly below that intended, and this could account for an increased size. It is not worth while to discuss the causes of the variation until analysis has gone further. The varied size in the group experiments cannot be explained by the varied conditions in amounts of food, etc. (cf. Fig. 5); there is a great variation with standard amounts of

food. The tongue jars gave a considerably smaller variation in size than the jam pots. This suggests that there is no need for the deeper container.

SUMMARY

1. The highest temperature at which *Ptinus tectus* should be reared is near 24·7° C.

2. Humidities higher than 70 % cannot conveniently be used because of the growth of moulds. Lower humidities prolong development.

3. The best food tried was 95 % whole ground wheat with 5 % dried brewers' yeast.

4. The smallest quantity of this food which permits completion of development is about 3 mg. per animal. This produces miniature adults, one-third of the normal weight, in almost normal time. About 30 mg. is required to produce full-sized adults. These statements apply to individuals reared in isolation.

5. When a group of several larvae are reared in one container, development is prolonged by up to 40 % (which does not occur at all with isolated animals even if food is so short as to cause severe stunting), while some diminution of size (20 %) is found. This is called the 'group effect'.

6. With groups of 8 larvae, as much as 5 g. of food per larva is required before the group effect disappears, 100 times as much as is needed for full and speedy development by an isolated larva.

7. With quantities of food per larva which are optimal or supra-optimal for isolated larvae, the group effect reaches its highest expression with 8 larvae in the group. It diminishes at smaller group sizes. Mass cultures show the effect to about the same extent as groups of 8.

8. Animals reared in groups show a wider range of variation in size than those reared in isolation, but their duration of development is not more variable.

9. No adequate explanation has yet been found for the group effect and no method has yet been found of producing from mass cultures animals as uniform in size as those reared singly.

10. Adults come into full and fertile lay about a week after emergence from the cocoon.

11. The rate of laying is markedly diminished if the animals are not given fresh food and water to drink daily.

12. A female can live for a year and lay nearly a thousand eggs.

13. In starting mass cultures, it is best to allow a large number of animals in good condition to lay for a day or so. No better result will be obtained by leaving the animals longer.

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PERIPHERAL NERVE AND REGENERATION IN CRUSTACEA

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(*Received* 6 *February* 1945)

There is now good evidence that in the Vertebrata peripheral nerves exert an important trophic effect on the regeneration of the structures which they innervate (Millot, 1931; Needham, J. 1942; Hamburger, 1944). In invertebrates the nervous system is not always necessary; Millot (1931, p. 117) concluded that in types where regenerative powers are comparatively limited (e.g. Mollusca, some Nemertinea), the nervous system is necessary, while in types with extensive regenerative powers (Tunicata, many Platyhelmia) it may be unnecessary. Some of the Crustacea are outstanding among the higher invertebrates for their extensive regenerative powers, and from the evidence available in 1927 (Korschelt, 1927, pp. 600–1) it seemed probable that in this group regeneration does not depend on the nerve supply. Subsequent publications have not materially affected this conclusion. In the present experiments evidence on the question is sought from the regeneration of a pair of thoracic limbs in *Asellus aquaticus* (L.).

METHOD

The limbs of the 7th thoracic segment were chosen for experiment; they are not specialized in any way and thus may be regarded as typical of the thoracic limbs in general. One of the pair served as a control, regenerating under normal conditions after amputation, under light ether anaesthesia, at the normal autotomy plane. The other limb was similarly amputated at the same time, and the nerve to the stump was severed where it passes from the body to the coxa, close to the ventral body wall, about $\frac{1}{3}$ mm. proximal to the level of amputation. The damage to the superficial tissues was not great under these conditions; visible haemorrhage ceased within half a minute and the wound cicatrized readily. A trace of proflavine was used in the water as an antiseptic both during operation and subsequently. Right and left limbs were used equally for experiment, to allow for any possible intrinsic difference between the two in their rate of regeneration (Needham, 1943, p. 57). In a parallel series of operations, serving as additional control, a wound equal in magnitude to that caused by denervation was inflicted on the base of one limb from the dorsal surface. In neither series was the blood supply to the regeneration blastema directly affected by the operation, so that the second series may be regarded as a suitable control for the effect of mere tissue destruction in the limb base. Microscopical sections indicated that, in general, the dorsal wound caused more widespread damage than the ventral denervation, since muscle tissue was largely affected; the muscles concerned subsequently degenerated along their length, often remote from the initial lesion.

The regenerating limbs were measured, as in a previous paper (Needham, 1943), on their first appearance externally, usually at the first post-operational ecdysis (scale 56 units = 1 mm.). The significance of the mean control-experimental (C.-E.) difference in total length of the regenerate was tested statistically (Tables 1, 2). In both series of experiments data were obtained for twenty-one individuals, representing a survival of about 37% in the denervation series and 66% in the other. In three individuals of each series the experimental limb had not appeared externally when the control was measured. These zero figures increase the significance of the mean C.-E. difference in the type of test used, more than is perhaps justified (Table 3), and they were therefore omitted in making the crucial tests. The test adopted was that the mean C.-E. difference should be more than 3× its probable error.

RESULTS

Denervation under the conditions of this experiment caused a significant retardation of regeneration (Table 1). The significance is not masked by individual variations in rate of regeneration and in the time interval between operation and measurement of the regenerates (p. 146). An equivalent wound not affecting the peripheral nerve may also have a retarding effect but was scarcely significant (Table 2), even when including the three individuals in which there was no external regeneration of the experimental limb (Table 3).

No evidence was obtained concerning the nature of the particular functional component of the peripheral nerve which affects regeneration of these limbs. The effect may be purely trophic, since no marked abnormalities in the form of the regenerates were obtained.

The results suggest not only a difference between the mean length of experimental and of control limb in each of the two series, but also a difference in the mean length of the control limb between the two series. The various mean lengths were as follows:

	Denervation series		Dorsal wound series	
	Experimental	Control	Experimental	Control
For 18 individuals	112·7	170·0	145·7	155·7
For 21 individuals	96·6	172·2	124·9	156·3

The shorter experimental limb of the denervation series is correlated with the longer control limb, suggesting the possibility that regeneration of the control limb may be accelerated in proportion to the degree of retardation of its experimental partner. The difference between the means of the two controls is, however, not statistically significant either when using actual limb lengths or relative limb lengths (limb length/body length) as the basis for the tests. The difference is more nearly significant when estimated by the first method, which is no doubt partly explained by the slightly greater mean body length in the denervation series (8·1 mm. compared with 7·9 mm.). Nevertheless, some support for the above hypothesis of competition between the two limbs of a pair of regenerates was provided by the great length of the control limb in four of the six individuals with a zero measurement for the experimental limb (see also p. 146 and Needham, 1943, p. 59).

Table 1. *Effect of denervation on regeneration of one of the 7th pair of thoracic limbs of* Asellus aquaticus

No.	Experimental limb length mm. × 56	Control limb length mm. × 56	Difference C.-E.
1	15·0	89·6	74·6
2	162·9	228·7	65·8
3	103·4	99·5	−3·9
4	86·6	204·1	117·5
5	103·9	179·8	75·9
6	48·5	106·1	57·6
7	153·0	177·5	24·5
8	78·7	91·3	12·6
9	128·7	188·3	59·6
10	187·6	221·7	34·1
11	131·2	185·8	54·6
12	210·4	240·4	30·0
13	63·3	164·4	101·1
14	98·8	130·8	32·0
15	142·5	175·7	33·2
16	66·9	119·3	52·4
17	175·1	219·0	43·9
18	72·9	237·1	164·2
			+1029·7
	Mean difference =		57·2
	Probable error of mean =		6·36
19	0·0	153·8	
20	0·0	200·6	
21	0·0	202·9	

Table 2. *Effect of comparable wound, not affecting nerve, on regeneration of the 7th pair of thoracic limbs of* Asellus aquaticus

No.	Experimental limb length mm. × 56	Control limb length mm. × 56	Difference C.-E.
1	187·0	188·3	1·3
2	187·4	193·8	6·4
3	56·9	92·0	35·1
4	192·9	205·8	12·9
5	128·5	126·4	− 2·1
6	226·0	260·4	34·4
7	120·6	128·6	8·0
8	108·8	105·1	− 3·7
9	132·6	72·7	−59·9
10	121·0	134·3	13·3
11	123·3	130·0	6·7
12	143·8	164·5	20·7
13	106·7	146·5	39·8
14	130·5	128·8	− 1·7
15	117·6	177·2	59·6
16	168·1	171·2	3·1
17	238·1	246·0	7·9
18	133·6	130·9	− 2·7
			+179·1
	Mean difference =		9·95
	Probable error of mean =		3·93

Table 3. *As Table 2 but including three individuals with zero measurements for experimental limb*

No.	Experimental limb length mm. × 56	Control limb length mm. × 56	Difference C.-E.
19	0·0	70·0	—
20	0·0	176·6	—
21	0·0	232·2	—
	Mean difference C.-E. =		31·33
	Probable error of mean =		9·40

DISCUSSION

The absence of qualitative abnormalities in the present experiments is not complete proof that the nerve exerts only a trophic effect, as in Vertebrata (p. 144). The limbs were always reinnervated by their original, appropriate nerve, which, if it determines the nature of the regenerate, will no doubt usually restore the *status quo*. The classical experiments of Herbst (1896) strongly indicated that the nerve ganglion determined the nature of the regenerate: that this effect may be indirect (Child, 1941, pp. 342–3) does not alter the main fact. Previous tentative suggestions that the nervous system may determine morphology (Needham, 1941, pp. 79–81; 1943, p. 69) merit further investigation.

It may be necessary to revise Millot's rule (p. 144) ventually. The nervous system would seem to be

important for regeneration in two groups, the Crustacea and the Amphibia, with relatively extensive powers of regeneration, and the correlation would seem to be a direct one, between the importance of the nervous system for regeneration and the complexity of organization of the animal rather than an inverse correlation between the former and relative power of regeneration.

The results of the dorsal wound series suggest that any tissue damage proximal to the level of amputation may retard regeneration to some extent. This might be due to competition for materials essential to regeneration (cf. p. 145), since neither nerve nor blood supply was affected. In the denervation series, however, the factor of competition probably did not play any significant part; microscopical sections indicated that no appreciable regeneration occurred until the blastema was reinnervated, by which time repair of the damage due to the operation of denervation was practically complete.

It is important that statistically significant differences can be demonstrated in these experiments in spite of the difficulty of obtaining strictly comparable measurements in the different individuals. No accurate measurements can be made until after the ecdysis at which the regenerate appears externally, and this occurs a very variable time after amputation, even in individuals of the same size and when amputation is performed at a fixed interval after the previous ecdysis; thus the measured length of the control limb shows considerable individual variation. It is possible to offset this variation to some extent by using the *percentage* C.-E. differences in the tests for significance rather than the *actual* differences of Tables 1 and 2, and such tests do show an increased significance. Since the curve of regeneration is sigmoid and not linear, however, the percentage C.-E. difference is not a strictly comparable estimate, at all stages of regeneration, of the difference between the two regenerates. In view of the unavoidable variables it was decided not to restrict the experiments to individuals of one particular body size. It seems probable that, with precautions to minimize all variables, statistically significant results might generally be expected in experiments on regeneration in the Crustacea.

In the denervation series, but not in the other, the difference between the mean experimental limb length and the mean control limb length is found to be less significant than the mean C.-E. difference. This probably indicates that the variance of C.-E. is less than that of control or experimental themselves, and that denervation arrests regeneration completely for a certain time after which it proceeds at about the same rate as in the control limb. This is supported by microscopical examination of denervated blastemas (see above). If, on the contrary, the effect of denervation were to reduce the rate throughout the period of regeneration then the C.-E. difference might be expected to vary as much as control and experimental themselves.

SUMMARY

1. The peripheral nerve appears to have an important trophic influence on the regeneration of thoracic limbs in the crustacean *Asellus aquaticus*, and section of the nerve proximal to the level of amputation causes a significant retardation of regeneration.

2. The retardation is statistically demonstrable in spite of the number of variables affecting the rate of regeneration.

3. Regeneration is probably completely inhibited until the blastema becomes reinnervated by the regenerating nerve.

4. Comparable damage to the limb base, not affecting the nerve, may also cause some retardation of regeneration, although, in these experiments, this was not clearly significant.

I am greatly indebted to Prof. H. S. Raper for the facilities I have enjoyed in carrying out this work, to Prof. J. Gray for his helpful advice on the manuscript, to Dr G. L. Camm for assistance with the statistical tests, and to my wife for her continued help throughout.

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STUDIES ON THE ORGANIZER PROBLEM IN *PELMATOHYDRA OLIGACTIS*

I. THE INDUCTION POTENCY OF THE IMPLANTS AND THE NATURE OF THE INDUCED HYDRANTH

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(*Received* 8 *February* 1945)

(With Three Text-figures)

The existence of a physiological gradient in *Hydra* has been repeatedly demonstrated by a number of investigators, and the hypostome, which is supposed to be the region of highest metabolic activity, has generally been regarded as the organization centre of the animal. Earlier transplantation experiments (Browne, 1909; Rand, Bovard and Minnich, 1926; Mutz, 1930) indicated that when a piece of hypostome is grafted, it can act as an organizer and induce a new hydranth. Although these are all well-known facts, the underlying mechanism in the induction process remains obscure. The present work is the first step in an attempted physiological analysis of the problem, with special reference to the induction potency of implants derived from different body levels, and to the nature of the induced hydranth.

MATERIAL AND METHODS

The common brown *Hydra*, *Pelmatohydra oligactis*, was used in this investigation. The specimens were kept in the laboratory in glass aquaria containing well water and some *Hydrilla* plant, and fed regularly on *Daphnia*. The technique was that of homoplastic transplantation, using only uniform non-budding animals, which had not been fed for 2 or 3 days, as hosts and donors respectively. The grafts were pieces taken from different body levels of a *Hydra* sectioned in the manner shown in Fig. 1, and stained supravitally with nile blue sulphate before implantation. Without exception grafts were made in the middle region of the body. After the take of the implants, the operated *Hydras* were cultured individually in porcelain dishes containing sterilized tap water changed at regular intervals, and kept in the dark at room temperature. Observations were made on alternate days following implantation, and the fate of the implants was recorded.

EXPERIMENTAL RESULTS

(1) *Transplantation experiments with a hypostome fragment as graft*

Pioneer work of Browne (1909) had demonstrated that a new hydranth is produced when a small piece of hypostome is grafted into another individual, and that the new individual determined by the graft is derived to a considerable extent from the host body. Later, Rand *et al.* (1926), and especially Mutz (1930), performed similar experiments. They did not, however, consider the constitution of the induced hydranth. In the present cases, the demarcation between the stained graft and the unstained host tissue was so sharply defined that the determination of the constitution of the induced hydranth was a matter of ease. It was found that, in the majority of cases, the

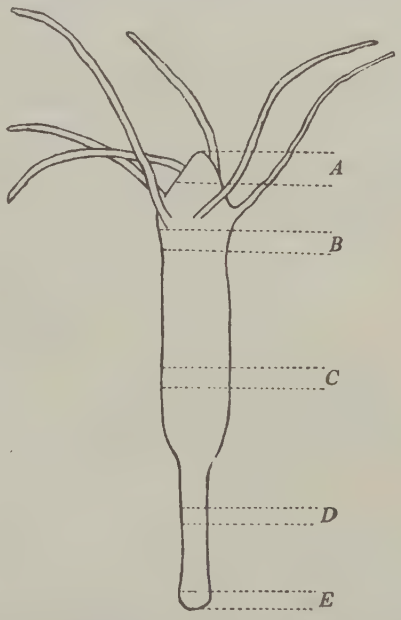

Fig. 1. A semidiagrammatic sketch showing the planes of section for different kinds of grafts used in the transplantation experiments. *A*, hypostome; *B*, subtentacular region; *C*, middle body region; *D*, stalk region; *E*, pedal disk.

hypostome of an induced bud was supplied by the implant itself, and the rest, including the tentacles and the body proper, was derived from the host, as shown in Fig. 2. Sometimes, however, the induction process was accompanied by a certain degree of self-reconstitution of the implant, giving an induced bud, with tentacles formed at the expense of both host and implant materials. Table 1 summarizes some of the results thus far obtained.

induction nor self-reconstitution has yet been recorded.

(3) *Transplantation experiments with a fragment from the middle of the body as graft*

The fate of implants obtained from this level of section fell into two groups. They were either assimilated by the host, becoming a part of its body wall, or reorganized themselves into new hydranths

Table 1. *The fate of implanted one-quarter hypostome fragments of* Pelmatohydra oligactis *grafted into the middle region of the host body of an individual of the same species*

Exp. series	No. of cases	Fate of implant (in number of cases)				% of induction
		Assimilated	Transformed into a single tentacle	Induction of new hydranth		
				Typical induction	Induction accompanied by self-reconstitution	
1	16	2	—	12	2	87
2	13	2	1	7	3	77
3	16	—	2	13	1	87

Table 2. *The fate of implanted fragments from the subtentacular region of* Pelmatohydra oligactis *grafted as indicated in Table 1*

Exp. series	No. of cases	Size of implant	Fate of implant		
			Assimilated	Self-reconstitution	Induction of new hydranth
1	42	$\frac{1}{2}$ ring	42	—	—
2	10	$\frac{1}{4}$ ring	10	—	—

Table 3. *The fate of implanted fragments from the middle body region of* Pelmatohydra oligactis *grafted as indicated in Table 1*

Exp. series	No. of cases	Size of implant	Assimilated	Fate of implant		
				Self-reconstitution	Differentiated into a pedal disk	Induction of new hydranth
1	20	$\frac{1}{2}$ ring	18	—	2	—
2	25	$\frac{1}{4}$ ring	24	1	—	—
3	16	$\frac{1}{6}$–$\frac{1}{8}$ ring	15	1	—	—

From the table, it can be seen that a piece of hypostome one-fourth of the whole in size will be able to give, on an average, 84% inductions, most of which are typical. In spite of the relatively small number of cases, therefore, it seems safe to say that under experimental conditions the implant is a highly potent system, in most instances able to induce the formation of a new hydranth.

(2) *Transplantation experiments with a fragment from the subtentacular region as graft*

The results of transplantation experiments with this kind of graft are given in Table 2. The grafts were found to be assimilated in all cases. Neither

through a process similar to the reconstitution of an isolated fragment. Such a reconstituted hydranth, therefore, consists of implant materials only. In a few exceptional cases (2 out of 61), the graft reorganized itself into a distinct pedal disk. Some of the data are presented in Table 3.

(4) *Transplantation experiments with stalk as graft*

Animals were first sectioned along plane *D* in Fig. 1 to obtain rings of tissues, which were then bisected and used as grafts. The grafts taken were invariably found to be assimilated at an early or later period. In accordance with their inability to regenerate (Goetsch, 1920; Weimer, 1928), no self-

reconstitution of such grafts has yet been observed. Sometimes the implant remained as a well-defined outgrowth showing pedal characters. Furthermore, owing to the readiness with which they disintegrate after bisection, it was very difficult to get a sufficient number of satisfactory grafts. Out of a total of twenty-four experiments so far performed, the implants were assimilated in twenty-two cases, and differentiated themselves into pedal disks in the remaining two.

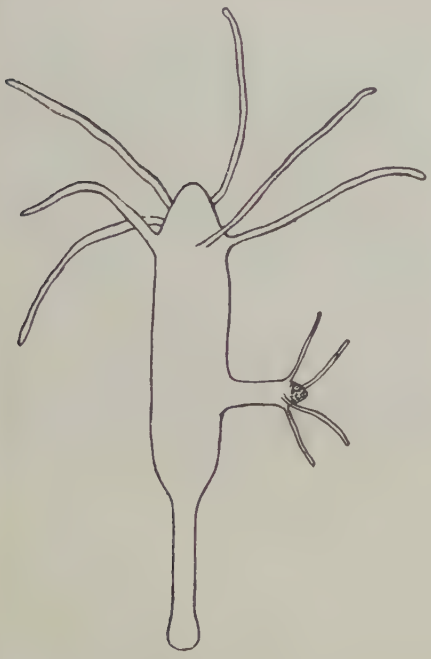

Fig. 2. A sketch showing typical induction. Note that the dotted implant material is confined to the hypostome region of an induced bud, while the tentacles and the body proper of the latter are all supplied by the host tissues.

(5) *Transplantation experiments with pedal disk as graft*

The pedal disk of *Hydra* has the characteristic properties of mucus secretion and gas production. When pieces of it (one-half to one-fourth of the original size) were grafted in, and taken by, the host, they were generally not assimilated; on the contrary, their characteristics remained unchanged for 3–5 weeks or even longer. Thus, in only three out of thirty-seven experimental individuals were the implants eventually absorbed; the rest were each provided with two pedal disks, indistinguishable in all respects except that of size (Fig. 3).

DISCUSSION

The presence of an 'axial gradient' in fresh-water *Hydra* is suggested by gradated properties of various kinds, such as the rate of reconstitution of isolated fragments (Weimer, 1928), the rate of disintegration in cyanide (Child & Hyman, 1919) and the rate of methylene-blue reduction (Child, 1934). Recently, a gradient in tentacle development was reported (Rulon & Child, 1937). In view of these well-established facts, it was of considerable interest to test whether or not there is a correlation between

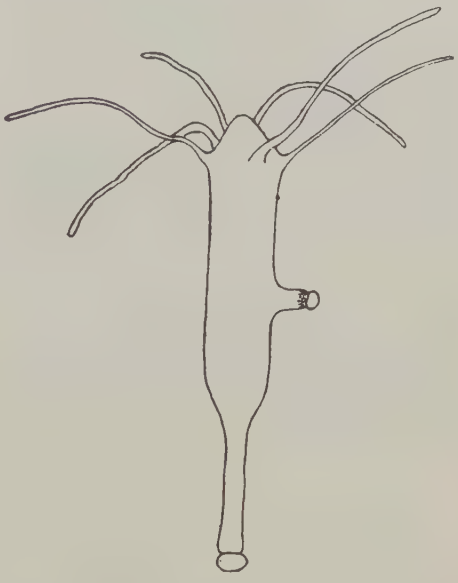

Fig. 3. A sketch showing that the characteristic properties of a pedal disk are retained after transplantation. The implant secretes a gas bubble just as does the pedal disk of the host.

the phenomenon of induction and the physiological gradient. If the induction potency were also a gradient character, then it might be revealed by transplantation experiments in terms of relative frequency of induction. From the data given here, however, it may be concluded that such a gradient of induction potency does not exist: induction ability is confined to the cells of the hypostome. A typical induced bud is composed of hypostome derived from the implant itself while the rest originates from the host tissues. Not infrequently, however, the implant materials also give rise to tentacles. This is understandable since the ability of an isolated hypostome fragment to give complete regeneration under favourable conditions has been proved.

Assimilation is found to occur most frequently when a piece of stalk is used as implant. In a few cases the differentiation of the graft into a pedal disk may be related to the suggestion of Burt (1925) that the pedal region of a *Hydra* has its own unipotent system. A similar fate of assimilation may befall grafts derived from the middle as well as from the subtentacular region. In the experiments undertaken thus far, no induction could be obtained with these grafts, and self-reconstitution occurred only rarely. These results lead to the conclusion that assimilation takes place more easily if the graft is composed of tissues the potency of which is the same as, or at least very similar to, that of the host tissues in the region of implantation. Moreover, the assimilation process can, perhaps, best be explained by the regulative action of the host.

The fact that implanted pedal disks appear to retain their own characteristics clearly indicates that the disk is composed of a group of specifically differentiated cells. Furthermore, its low frequency of assimilation seems to show that the differentiation process is irreversible. The incapacity of a piece of pedal disk to perform any induction agrees with the fact that in isolated culture such pieces are unable to regenerate new *Hydras* and may disintegrate within 10 days.

SUMMARY

1. Homoplastic transplantation in *Pelmatohydra oligactis* has been studied with implants obtained from different body levels.

2. As a result of the regulation of the host, assimilation of the graft has been recorded in all cases. The frequency of assimilation is higher when graft and host are composed of cells having the same (or nearly the same) developmental potentiality.

3. Pedal disk is usually not assimilated, but maintains its own properties following implantation.

4. No gradient of induction potency can be demonstrated in this animal. Perhaps owing to unknown intrinsic factors, the induction power is limited to the cells of the hypostome.

5. A typical induced hydranth is always composed of hypostome supplied by the graft itself, and of tentacles and body derived from the host.

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STUDIES ON THE ORGANIZER PROBLEM IN *PELMATOHYDRA OLIGACTIS*

II. THE EFFECT OF SOME RESPIRATORY INHIBITORS AND STIMULANTS AND OF OXYGEN DEFICIENCY ON THE INDUCTION POTENCY OF THE HYPOSTOME

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(*Received* 8 *February* 1945)

In a previous communication (Yao, 1945(*a*)) it has been shown that only the material of the hypostome, when used as implant, has the properties of an organizer; such a graft is able to induce a new hydranth, in which the hypostome is developed from the implant itself, while the rest, including tentacles and body proper, is derived from the host. Supposing the induction process to be evoked by some active substance present in the organizer, this active substance, as well as the initial act of induction, might be modified by those agents which inhibit or stimulate respiration. The present paper deals with the effect of certain respiratory inhibiting and stimulating agents on the induction ability of the hypostome. The effect of oxygen deficiency on induction will also be described.

The details of the method used will not be described, since these were essentially similar to those given in the former paper.

EXPERIMENTAL RESULTS

(1) *The effect of potassium cyanide*

The effect of potassium cyanide has been tested in two ways. In the first method, hypostome taken from a donor was cut into four pieces. Two were treated with $2{\cdot}5 \times 10^{-3}$ *M* potassium cyanide for 1 min. before implantation, while the untreated two served as controls. The concentration of potassium cyanide and the duration of treatment were so chosen that no disintegration of the implant occurred. From the data given in Table 1 it is clear that the treated implants can still give about 86% induction, a value equal to that of the controls.

In the second method, the donors were first immersed in potassium cyanide solution for 30 min., and their hypostomes subsequently used as implants. Some typical experiments at concentrations of $2{\cdot}5 \times 10^{-3}$ *M* and 5×10^{-3} *M* respectively are shown in Table 2. Obviously, treatment with potassium

Table 1. *The effect of potassium cyanide on the induction potency of one-quarter hypostome fragments. Concentration* $2{\cdot}5 \times 10^{-3}M$; *time of treatment,* 1 *min.*

No. of cases		No. of take	Fate of implant			% of induction
			Induction of new hydranth	Transformed into a single tentacle	Assimilated	
Control	32	30	26	1	3	87
Experimental	32	29	25	2	2	86

Table 2. *The effect of potassium cyanide on the induction potency of hypostome. Donor immersed in potassium cyanide solution for* 30 *min.*

Concentration	No. of cases	Fate of implant				% of induction
		Assimilated	Transformed into a single tentacle	Induction of new hydranth		
				Typical	Atypical	
$2{\cdot}5 \times 10^{-3}$ *M*	15	1	—	13	1	—
	15	3	1	11	—	—
	25	2	—	23	—	—
	15	3	—	10	2	—
Total	70	9	1	57	3	86
5×10^{-3} *M*	31	3	—	16	12	—
	13	4	—	3	6	—
	17	4	—	10	3	—
Total	61	11	—	29	21	82

cyanide in these high concentrations did not produce any significant change in the percentage of induction.

As may be seen from the table, however, there is a marked increase in the number of atypical inductions after treatment with 5×10^{-3} *M* potassium cyanide, a fact which is, as yet, difficult to explain. In some cases, transplantation has also been performed with implants obtained from donors which had remained in $2{\cdot}5 \times 10^{-3}$ *M* potassium cyanide for 1–24 hr. The results thus obtained showed no difference from those already mentioned; the induction power of about 85% of the implants was still unaffected.

finally adopted for treatment. The tentacles of a *Hydra* immersed in this solution will have partially disintegrated within 15 min. In Table 4 is given the result of transplantation with implants taken from donors treated in this way. As is the case after lithium chloride treatment, the induction ability of the hypostome, if influenced at all, is influenced in an all-or-none manner, since the hydranths induced are nearly always typical; but the percentage of implants assimilated or disintegrated is increased. This latter effect is responsible for the lowering of the induction percentage. Isolated hypostome pieces have not been treated by this agent.

Table 3. *The effect of lithium chloride on the induction ability of hypostome*

Treatment	No. of cases	Fate of implant					% of induction
		Disintegrated	Assimilated	Transformed into a single tentacle	Induction of new hydranth		
					Typical	Atypical	
Isolated hypostome fragment	Control 32	—	8	—	24	—	72
	Experimental						
1×10^{-2} *M*, 1 min.	30	—	6	4	24	—	67
Intact hypostome, 30 min.: 2×10^{-2} *M*	34	8	4	—	17	5	65
1×10^{-2} *M*	36	6	4	—	26	—	72

Table 4. *The effect of sodium fluoride on the induction ability of hypostome. Concentration* $2{\cdot}5 \times 10^{-2}$ *M; time of treatment,* 15 *min.*

Exp. series	No. of cases	Fate of implant				% of induction
		Disintegrated	Assimilated	Induction of new hydranth		
				Typical	Atypical	
1	16	3	5	8	—	—
2	19	5	3	10	1	—
3	17	4	4	9	—	—
4	12	3	3	5	1	—
5	16	4	3	9	—	—
Total	80	19	18	41	2	54

(2) *The effect of lithium chloride*

Similar experiments have been made using lithium chloride instead of potassium cyanide. Whether treated before or after sectioning, we found a lowering of the total number of cases showing induction by 15–20% (Table 3). This lowering was largely due to the higher percentage of assimilation and disintegration after implantation. In those cases where the implant did not disintegrate or undergo assimilation, induction of a new hydranth proceeded quite normally.

(3) *The effect of sodium fluoride*

In testing the effect of sodium fluoride, a series of concentrations ranging from 5×10^{-2} to $2{\cdot}5 \times 10^{-3}$ *M* was used. A concentration of $2{\cdot}5 \times 10^{-2}$ *M* was

(4) *The effect of methylene blue*

In view of its ability to act as a hydrogen donator or acceptor methylene blue may serve as a respiratory catalyst. Numerous data have been collected on the increase in oxygen consumption of a living cell after appropriate treatment with this reagent. Preliminary experiments showed that pretreatment with methylene blue in concentrations from 1 : 10,000 to 1 : 100,000 did not produce any notable effect on the hypostome as regards the percentage of induction. Similar experiments were made with fragments derived from the subtentacular region. Rings of tissue were cut out and divided into pieces of size suitable for implantation. They were subsequently treated with 1 : 10,000 methylene blue for 5–10 min. As the

dye stained the implant with considerable intensity, no further vital staining with nile blue sulphate was necessary. Results thus far obtained are given in Table 5.

Table 5. *The effect of methylene blue on the fate of implanted fragments derived from subtentacular region. Concentration,* 1 : 10,000*; time of treatment,* 5–10 *min.*

Exp. series	No. of cases	Fate of implant		% of induction
		Assimilated	Induction of new hydranth	
1	23	23	—	—
2	22	22	—	—
3	24	22	2	—
4	14	13	1	—
Total	83	80	3	4

Instead of invariable assimilation, as found with untreated fragments, in three out of eighty-three cases treated with methylene blue, typical induction occurred. A possible interpretation of this result will be given later on.

(5) *The effect of oxygen on hypostome induction*

From the experimental results just given, it seems logical to conclude that the induction potency of hypostome is not drastically modified by either respiratory inhibitors or stimulants. In other words, the normal action of the induction substance present in the organizer is not so essentially related to the principal cellular respiratory system that inhibitors or stimulants of respiration profoundly change the percentage or the type of inductions obtained. The next point worthy of investigation is the effect of oxygen on the induction process. Hypostome transplantation was repeated under anoxic conditions. The operated *Hydras* were placed individually in finger bowls and covered with a minimum but sufficient amount of water which was freshly prepared by prolonged boiling, to expel all dissolved air, and cooling without any disturbance. These were then quickly placed in a small, air-tight vessel already supplied with the calculated amount of KOH-pyrogallol mixture. It was not certain that all oxygen was absorbed in this way, but from the fact that all the *Hydras* exhibited a gradual resorption of their tentacles and body proper after 72 hr., as do physiologically depressed individuals, it was certain that the medium contained very little, if any, oxygen. Hypostome fragments under these conditions were still found to be active in induction. Among the 60–70 % of induced cases, two-fifths gave typical inductions, while the remaining three-fifths induced only tube-like outgrowths. These induced buds, whether they had tentacle rudiments or not, all showed poor development, presumably because of the lack of oxygen.

DISCUSSION

Potassium cyanide effectively inhibits cellular respiration through its poisoning action on the Keilin-Warburg system. Both isolated and intact hypostomes have been treated with this reagent at concentrations higher than those used to inhibit respiration. Since such hypostome fragments induce new hydranths in as typical a manner as the controls, it is clear that the effectiveness of any induction substance within the cells of the hypostome is not profoundly changed by this respiratory inhibitor.

Lithium chloride and sodium fluoride are also respiratory inhibitors, though they produce their effect through disturbance of carbohydrate metabolism. Treatment with these reagents at rather higher concentrations than those necessary for the inhibition of respiration does not suppress the ability of the hypostome to induce typical hydranths. The decrease in the percentage of inductions is associated with the more ready disintegration and assimilation of the treated implants as compared with the controls.

In agreement with these results, methylene blue has no obvious action on the active substance of the hypostome. Implants from the subtentacular region, however, which normally are always assimilated by the host after implantation, occasionally show induction after pretreatment with methylene blue. Though the number of cases observed is very small the reality of the effect is fairly certain, since the chance of contamination with a trace of hypostome material is remote. A similar phenomenon has been observed by Waddington, Needham and Brachet (1930) in their extensive studies on the nature of the amphibian organization centre. They demonstrated that the power of performing an induction can be evoked in the ventral ectoderm by the action of methylene blue. Since the metabolism of the hypostome and other body regions in *Hydra* has not yet been studied, nothing is known of the mechanism involved in this phenomenon in *Hydra*. It is possible, however, that the cells of the subtentacular region may contain some inactive precursor of the inducting substance which can be activated by methylene blue. Further experiments on this point are now in progress.

The success of induction under conditions of oxygen deficiency suggests that the initial act of induction is not dependent on the presence of oxygen in normal concentration. This agrees fairly well with the experimental results of Brachet (1939), who has presented evidence that the induction of amphibian neural tube can take place under anaerobic conditions and with explants treated with cyanide.

SUMMARY

1. The induction ability of the hypostome of *Pelmatohydra oligactis* is not drastically modified by respiratory stimulants or inhibitors such as methylene blue, potassium cyanide, lithium chloride and sodium fluoride, under experimental conditions.

2. A pretreatment with methylene blue may confer induction power on the normally inactive subtentacular implant.

3. Induction does not seem to be closely linked to the cyanide-sensitive respiratory system.

4. Oxygen is possibly of secondary importance only in the initial act of induction. Its absence, however, inhibits the further development of an induced bud.

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STUDIES ON THE ORGANIZER PROBLEM IN *PELMATOHYDRA OLIGACTIS*

III. BUD INDUCTION BY DEVELOPING HYPOSTOME

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(*Received* 8 *February* 1945)

(With Two Text-figures)

INTRODUCTION

From the standpoint of a developmental physiologist, budding involves the physiological isolation from parental dominance of a portion of undifferentiated tissue, its subsequent differentiation into a new individual and, finally, the separation of the latter from the parent. This means that the chief process involved in budding is cellular differentiation. *Hydra* is a very suitable object in which to study the general problem of differentiation from this angle, since asexual budding is frequent and the process of differentiation relatively simple. In spite of the voluminous literature dealing with regeneration and transplantation in *Hydra*, experiments on these lines are few. Rulon & Child (1937) have recently investigated the developmental pattern in the same animal. Their study was chiefly concerned with the origin of tentacular pattern, the early determination of the bud and the interrelation between an actively growing bud and its parent. Since we are interested in the organizer problem of the animal, our investigation will deal mainly with the differentiation of the hypostome. In a previous communication, one of us (Yao, 1945(*a*)) has shown that there is no gradient in induction potency, that is to say, induction potency is a property peculiar to the hypostome cells. We may use this fact to detect the invisible differentiation of the hypostome within a developing bud, since success in the induction of a new hydranth by a bud fragment will indicate the appearance of the hypostome within the bud. In the present paper, evidence will be presented which suggests, first, that the determination of the hypostome occurs very early in bud development, and secondly, that the mode of induction by a developing hypostome is different from that of an adult hypostome.

MATERIAL AND METHOD

Hydras in good condition and with a single bud were selected from the flourishing stock to serve as donors. The buds were usually in various developmental stages. The extreme tip of a developing bud was then excised under lower-power magnification by a single cut with a sharp scalpel, stained with nile blue sulphate and implanted into a healthy but non-budding *Hydra* from the same stock. Transplantation technique similar to that previously described was used in the present investigation. The operated *Hydras* were classified into groups according to the developmental condition of their implant and cultured individually in porcelain dishes containing sterilized well water. Observations were made day by day following implantation. Both the fate of the implant and the manner of induction (if the reaction was positive) were carefully recorded.

MORPHOLOGICAL OBSERVATIONS

Before giving a detailed account of the experimental data, it is necessary to summarize the observed morphological changes in a developing bud. From a close examination of the budding process in some thirty individuals, we arbitrarily distinguish six separate and successive stages in bud development. The first stage shows merely a localized outgrowth of the body wall in the budding zone. In the next, stage 2, the bud becomes more prominent and the distal end becomes pointed. This stage seems to be related to the invisible determination of tentacles and body proper. The appearance of visible tentacle rudiments marks the beginning of the third stage. Further growth of the body and of the tentacles leads to stage 4, at the end of which the tentacles are still non-motile. As the bud enters stage 5, its hypostome and tentacles become functional, so that occasionally the young polyp is able to feed on *Daphnia*. In the meantime, differentiation of the stalk is completed. In the last stage, the full development of a pedal disk involves the final separation of the young individual from the parent. These stages are represented in Fig. 1, *1–6*.

In their studies on tentacular pattern in the same animal, Rulon & Child (1937) also made observations on bud development. No special effort to compare their results with ours has been made, but

our first five stages are in rough correspondence with those shown in Figs. 1, 3, 5, 9 and 12, respectively, of their paper. Stage 6 was not represented in their figures. It corresponds to a bud just detached from the parent.

According to this classification, the donors used in our transplantation experiments fell into six categories. In making the experiments, only the extreme distal tip of the hypostome was removed. The size of the implant varied according to the stage of development; for example, the developing hypostome taken from the bud of stage 1 was bisected, while those taken from all other stages were divided into four.

Fig. 1, *1–6*. Lateral aspect of successive stages in the development of the bud of *Pelmatohydra oligactis*.

EXPERIMENTAL RESULTS

The experimental results are presented in Tables 1–6. In these, the results of interaction between the host and the implant are grouped in four classes. In the first, the implant disintegrated on the second day and left no trace of its transplantation to the host. In the second, the implant was assimilated by the host, leaving a blue spot on the body wall. In the third, the implant transformed itself into a single outgrowth, which sometimes looked much like a tentacle. These three classes, however, contributed only a small percentage to the whole result. In the fourth class only did interaction lead to the production of a new hydranth. All these classes are grouped in the tables under the heading 'Fate of implant'.

The mode of induction by a developing hypostome was different from that by a mature one. For convenience' sake, the former kind of induction was tentatively termed type II induction, and the latter type I induction. The essential difference between these two types of induction was as follows: In type I, characteristic of mature hypostomes, one or two pairs of tentacles invariably grow out from the host within 24–48 hr. following implantation. The induction of the body proper may accompany the appearance of tentacles, but in most cases it occurs somewhat later. In type II induction, on the other hand, within 48 hr. of implantation only a tube-like outgrowth was induced; the tentacle rudiments generally became visible only after 72 hr. The differences between these two induction types are shown in Fig. 2, *1–4*. In either type the induced tentacles may be formed either entirely from host tissue (homotypic induction), or partially from the implant (heterotypic induction).

In Table 1, it is seen that, despite frequent assimilation of the implant (amounting to about 65 %), induction of a new hydranth by a hypostome rudi-

ment was possible in the first stage; this indicates that the hypostome may already be determined within the bud at this time. The results shown in Tables 2–4 are similar for each stage; over 80% of the implants produce new hydranths by type II inductions. Table 5 shows the result of transplantations with the bud tip in stage 5. At this stage a transition between the two types of induction occurs. Thus, out of a total of 108 cases, eighty-two gave inductions, of which 78% were still type II inductions, while the remaining 22% were distinctly type I inductions. This transition was almost complete in stage 6: out of eighty-five induced cases (Table 6), all but two gave type II inductions. This suggests that the full development of a bud in *Pelmatohydra oligactis* is always associated with an invisible change in its hypostome here made manifest in the sudden change in the mode of induction.

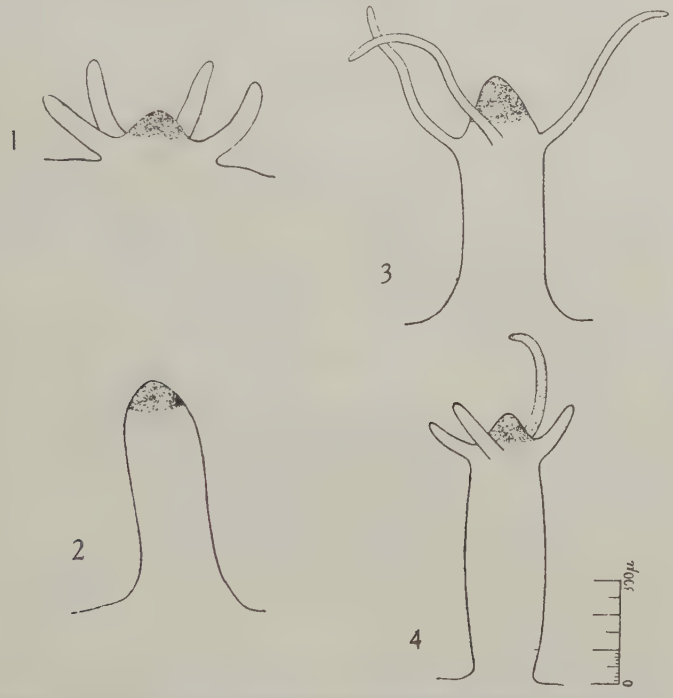

Fig. 2, *1–4*. Lateral aspect of induced bud, showing the distinction between the two types of induction. *1* and *2* show early stages in type I and type II induction, respectively. *3* and *4* show these same types at a later stage.

Tables 1–6. *The fate of implanted ½–¼ developing hypostome fragments of* Pelmatohydra oligactis *grafted into the middle region of the host body of same species*

Table 1. *Developing hypostome taken from bud in stage I*

Exp. series	No. of cases	Fate of implant			
		Assimilated	Transformed into a single tentacle	Induction of new hydranth (type II)	
				Homotypic	Heterotypic
1	14	6	0	5	3
2	28	17	1	7	3
3	34	25	2	1	6
4	14	11	0	3	0
Total	90	59	3	16	12
%	100	66	3	31	

Table 2. *Developing hypostome taken from bud in stage 2*

Exp. series	No. of cases	Fate of implant			
		Assimilated	Transformed into a single tentacle	Induction of new hydranth (type II)	
				Homotypic	Heterotypic
1	37	8	1	5	23
2	19	1	0	13	5
3	25	2	0	11	12
4	21	1	0	11	9
Total	102	12	1	40	49
%	100	2	1	87	

Table 3. *Developing hypostome taken from bud in stage 3*

Exp. series	No. of cases	Fate of implant			
		Assimilated	Transformed into a single tentacle	Induction of new hydranth (type II)	
				Homotypic	Heterotypic
1	26	3	1	15	7
2	26	2	0	20	4
3	19	4	1	9	5
4	15	1	0	8	6
5	18	2	0	9	7
Total	104	12	2	61	29
%	100	2	2	87	

Table 4. *Developing hypostome taken from bud in stage 4*

Exp. series	No. of cases	Fate of implant				
		Assimilated	Transformed into a single tentacle	Induction of an outgrowth	Induction of new hydranth (type II)	
					Homotypic	Heterotypic
1	16	5	0	0	0	11
2	26	1	0	1	18	6
3	13	3	0	0	4	6
4	33	5	1	0	6	21
5	18	1	0	0	6	11
Total	106	15	1	1	34	55
%	100	14	1	1	84	

Table 5. *Developing hypostome taken from bud in stage 5*

Exp. series	No. of cases	Fate of implant						
		Assimilated	Transformed into a single tentacle	Induction of an outgrowth	Induction of new hydranth			
					Type I		Type II	
					Homotypic	Heterotypic	Homotypic	Heterotypic
1	19	2	1	1	0	3	0	12
2	25	7	2	0	0	6	0	10
3	32	4	3	1	1	4	2	17
4	32	5	0	0	1	3	2	21
Total	108	18	6	2	2	16	4	60
%	100	7	6	2	17		60	

Table 6. *Developing hypostome taken from bud in stage* 6

Exp. series	No. of cases	Fate of implant							
		Assimilated	Transformed into a single tentacle	Induction of an outgrowth	Disintegrated	Induction of new hydranth			
						Type I		Type II	
						Homotypic	Heterotypic	Homotypic	Heterotypic
1	25	1	0	1	10	8	5	0	0
2	25	3	0	0	5	5	11	0	1
3	35	2	0	0	2	17	14	0	0
4	29	3	1	0	1	12	11	1	0
Total	114	9	1	1	18	42	41	1	1
%	100	8	1	1	16	73		2	

DISCUSSION

It has been shown by our transplantation experiments that the determination of the hypostome takes place very early in the development of a bud (assuming that this is synonymous with power to induce a new hydranth). The critical period lies probably in some phase of stage 1. This is not at all surprising in view of recent work by Rulon & Child (1937), who demonstrated, by an entirely different method, that the determination of body and stalk occurs at an early stage of tentacle development (stages represented by Figs. 1–3 in their paper). Since these 'very early buds' probably represent buds between stages 2 and 3 in our terminology, a still earlier determination of the hypostome as compared with other parts would be understandable in the light of the gradient concept. It seems reasonable to suppose that, during the development of a bud, hypostome is determined first, then tentacles, then body and so on.

It seems likely that certain undifferentiated cells within an incipient bud acquire the ability to induce new hydranths through some sudden change, the physico-chemical nature of which is as yet unknown. This idea is supported by facts described in the first two papers. On the one hand, it is found that a piece of undifferentiated tissue taken from or near the budding zone is invariably assimilated by the host when used as implant (Yao, 1945*a*). On the other hand, appropriate pretreatment of tissue taken from the subtentacular region with methylene blue may render it capable of induction (Yao, 1945*b*). It was therefore tentatively suggested that there may be present in the cells of the subtentacular region some inactive precursor of the induction substance which can be activated by methylene blue. We suggest now that an inactive precursor is also present in the undifferentiated cells of the budding zone, and that it is somehow activated during the first stage of bud development. This would agree with the fact (shown in Table 1) that the implant is sometimes active in induction but sometimes assimilated by the host (remembering that it is impossible to obtain a considerable number of buds in stage 1 having the same developmental history).

Various workers (Hyman, 1928; Weimer, 1928; Rulon & Child, 1937) have interpreted the phenomenon of budding in terms of the gradient concept, suggesting that it results from 'physiological isolation' from the dominant apical region. The demonstration of the characteristic mode of induction of a developing hypostome further indicates that the developing bud is, in an even stricter sense, completely isolated from the parent. When a part of the developing hypostome is implanted, its induction of a new hydranth follows the same course as is found in natural budding. This means that once the hypostome becomes irreversibly determined it acts as a new organization centre capable of controlling the subsequent development of the bud and of inducing a new bud when transplanted.

One more interesting point worthy of notice is the transition between the two types of induction which occurs in stage 5. It is clearly impossible to discuss the mechanism of this transition in general terms until the nature of induction is known, but if the idea of a precursor substance were correct, the entire picture might be as follows: The transformation of the inactive precursor into the active induction substance involves a chain of reactions, in the course of which an intermediate substance is produced, also capable of induction, but in an entirely different manner. This chain of reactions ends just before or after the detachment of a bud, so that hypostome derived from buds in stage 6 usually gives type I induction similar to an adult hypostome. On this view two substances of slightly different nature are responsible for the two types of induction. To test this possibility and in order to detect possible differences between these two hypothetical substances, further experiments on the effect of respiratory stimulants and inhibitors on the developing hypostome are now being carried out.

SUMMARY

1. Determination of the hypostome occurs very early in the development of a bud (our stage 1) in *Pelmatohydra oligactis*.

2. The mode of induction by a developing hypostome (type II) is different from that by an adult hypostome (type I). In the former case, an implant always induces a long tube-like outgrowth before any tentacle rudiment becomes visible. In the latter case, simultaneous induction of tentacles and body takes place. Frequently, tentacle rudiments appear before visible differentiation of the body.

3. Type II induction is characteristic of developing hypostomes from some phase of stage 1 up to a stage at which the bud possesses short, but non-motile tentacles (our stage 4).

4. As soon as the detachment of a young polyp from the parent occurs, there is a sudden change in the mode of induction, from type II to type I.

5. Possible explanations of the course of differentiation in the hypostome and of the transition between the two types of induction are suggested.

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THE OXYGEN AFFINITIES OF CERTAIN INVERTEBRATE HAEMOGLOBINS

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(*Received* 19 *April* 1945)

(With Two Text-figures)

For a variety of physiological and ecological reasons it is of importance to know the oxygen pressures at which the haemoglobins of different animals take up or part with oxygen. In the relatively few cases in which this has been studied in small animals, particularly invertebrates, the blood, since it is only available for investigation in very small amounts, has generally been considerably diluted with water. But the position of the oxygen dissociation curve of dilute haemoglobin is not necessarily the same as that in whole blood: the oxygen affinity may increase with dilution (Hill & Wolvekamp, 1936; Florkin *et al.*, 1941). To survey the oxygen affinities of various invertebrate haemoglobins I therefore devised a method of working with one or two drops of whole, or very slightly diluted, blood. The method is as follows.

The apparatus (Fig. 1), called a bulb-capillary, consists of a glass capillary (*a*) leading to a bulb (*b*) and a three-way tap (*c*). The rubber pressure tubing (*dd*) can be compressed by a screw (*e*) and leads to a tap (*f*). Before the pressure tubing is fitted to the capillary a rubber teat is attached to *g* and a drop or two of the blood to be studied is sucked into the bulb (*b*) through the bevelled orifice of the capillary. Great care is taken to avoid an air bubble on the drop of blood in the bulb as this retards or prevents subsequent equilibration of blood and gas. The teat is now removed, the pressure tubing, screw and tap (*d, e, f*) attached, and, with the apparatus horizontal, nitrogen, purified from residual oxygen in an electrically heated copper furnace (which reduced the oxygen content to 3 parts per million), is passed from *g* through the bulb until the haemoglobin is deoxygenated. If the bulb is occasionally warmed in the palm of one hand and the apparatus is shaken horizontally with the other hand while the nitrogen is streaming through it the deoxygenation is complete within 5 min. This is checked with a hand spectroscope, looking down through the bulb (*b*) into a mirror reflecting a lamp.

A suitable mixture of air and nitrogen, with or without carbon dioxide, is next passed from a gas burette through the bulb. The taps are then closed and the bulb-capillary is detached at *g* and rotated on a wheel in a water thermostat for half an hour, during which time the thin film of blood on the wall of *b* comes into equilibrium with the gas. The bulb-capillary is now removed from the water, dried, held vertically with the pressure tubing downwards, and some of the blood is drawn into the capillary (*a*) by manipulation of the screw (*e*).

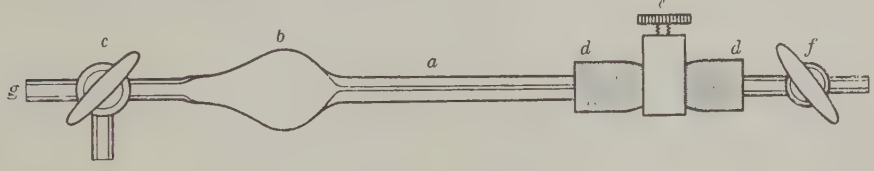

Fig. 1. Bulb-capillary: description in text.

The percentage of oxyhaemoglobin in the blood is then determined spectroscopically. With the bulb-capillary clamped in a horizontal position to a metal stand with a vertical rack and pinion movement, the horizontal filament of a 12 V., 24 W. car lamp is focused on the blood in the capillary, whence the light beam passes on into the side window of a Zeiss microspectroscope used as a differential colorimeter as described by Hall (1934) and Hill (1936). In this arrangement light from a second lamp passes up through two colorimeter cups, one above the other, before entering the microspectroscope from below. The plunger of the lower cup is attached beneath the upper cup, which can be moved vertically; by this means any optical mixture of the contents of the two cups is obtainable. The cups contain respectively dilute human oxyhaemoglobin and haemoglobin, the latter kept deoxygenated by sodium hydrosulphite in mildly alkaline solution.

Before the nitrogen was passed through the bulb-capillary at the beginning of the experiment, as described above, some of the blood to be studied had been drawn, by means of the screw (*e*), from the

bulb (*b*) into the capillary (*a*) and the concentration of oxyhaemoglobin in one of the colorimeter cups had been adjusted so that the α-bands in cup and capillary matched, the light intensities from the two lamps being equal. After equilibration of the blood with the gas mixture as described above the α-band in the capillary is matched with the appropriate optical mixture of oxy- and deoxyhaemoglobin in the spectrocolorimeter and thus the percentage saturation of the haemoglobin with oxygen is found.

After this the blood is screwed back from the capillary into the bulb, and, without the taps being opened, is re-equilibrated with the same gas mixture at another temperature. Thus a point on each of two oxygen dissociation curves, at different temperatures, is obtained. The taps are then opened and the blood is re-equilibrated with air and tested in the spectrocolorimeter to see that the α-band has not faded through haematin formation. If there is no fading the same blood can be used with another gas mixture.

and out of the trough; but this thickness gave too much light absorption by the cladoceran haemo-

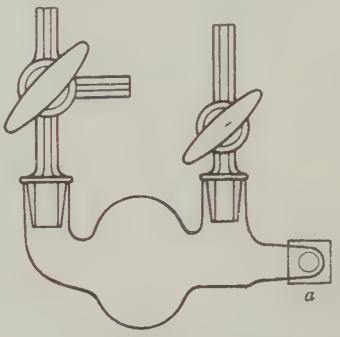

Fig. 2. Microsaturator with optical trough: explanation in text.

Table 1. *Oxygen pressures for half-saturation of haemoglobin with oxygen*

	Oxygen pressure (mm. Hg) for 50 % oxyhaemoglobin				Increase due to carbon dioxide	
	No carbon dioxide		1 % carbon dioxide			
	10° C.	17° C.	10° C.	17° C.	10° C.	17° C.
Chironomus riparius Mg.	0·5	0·6	0·5	0·6	× 1·0	× 1·0
Tubifex sp.	0·5	0·6	—	—	—	—
Ceriodaphnia laticaudata P. E. Müller	—	0·8	—	1·0	—	× 1·25
Homo sapiens L.	0·8	1·2	3·1	3·9	× 3·9	× 3·2
Thalassema neptuni Gaertner	0·9	1·4	1·4	1·8	× 1·6	× 1·3
Arenicola marina L.	1·5	1·8	2·0	2·6	× 1·3	× 1·4
Planorbis corneus L.	1·5	1·9	2·3	2·9	× 1·5	× 1·5
Daphnia magna Straus	2·0	3·1	3·0	4·9	× 1·5	× 1·6

Several bulb-capillaries were made, the capillaries having different diameters to suit bloods of different haemoglobin contents. In the cases of *Chironomus* larvae, *Arenicola* and *Planorbis*, whole blood was used, but with *Thalassema* and man the fluids were diluted 1 in 3 to haemolyse the cells. *Tubifex* and cladoceran haemoglobins were obtained by crushing the animals and clarifying the liquid with kieselguhr, etc., so these bloods were necessarily a little diluted by water adhering to the animals. The cladoceran blood was too poor in haemoglobin for the bulb-capillary and was studied in a special microsaturator (Fig. 2) designed so that after equilibration of blood and gas on the wheel in the water thermostat the apparatus could be turned through 90°, thus allowing the blood to flow down into the optical trough (*a*) for measurement of its oxyhaemoglobin percentage with the spectrocolorimeter. A difficulty in design arose in that the faces of the trough could not be made less than 9 mm. apart or the liquid would not flow into

globin. Consequently the blood, already somewhat diluted by water between the shell valves, had to be further diluted by about 50 %.

The blood or body fluid of each type was equilibrated with oxygen at one or more pressures chosen to give oxyhaemoglobin percentages close to 50. From the partial oxygen dissociation curve thus obtained the oxygen pressure for 50 % oxyhaemoglobin was deduced. The experimental data are given in the Appendix and the results in Table 1. Most bloods were studied at temperatures of 10 and 17° C., without and with carbon dioxide in a concentration of 1 % of an atmosphere. Table I shows that *Chironomus* and *Tubifex* have the highest, *Daphnia* the lowest affinity for oxygen. The value for *Chironomus* at 17° C. is of the same order of magnitude as that found by Leitch (1917). *Arenicola* agrees sensibly with the values of Barcroft & Barcroft (1924) and *Planorbis* with those of Macela & Seliškar (1925), although these two investigations were made with

diluted blood. The results of Florkin *et al.* (1941) with *Planorbis* whole blood differ greatly from mine. My values for man are lower than those of Hill & Wolvekamp (1936).

All the haemoglobins studied are seen to have a very high oxygen affinity compared with human whole blood at 37° C. and *p*H 7·4 (Hill & Wolvekamp, 1936) or with diluted *Spirographis* chlorocruorin at 20° C. and *p*H 7·7 (Fox, 1932), both of which are 50% saturated with oxygen at an oxygen pressure of 27 mm. of mercury. All the invertebrates studied live in situations which may lack oxygen, except *Thalassema* unless perhaps at low tide. *Ceriodaphnia* has a higher oxygen affinity than *Daphnia*: the former lives in fouler water.

The effect of acid on oxygen affinity is seen from Table I to be nil for *Chironomus* (cf. myoglobin, Hill, 1936) and considerable for man, while in the other animals studied 1% of an atmosphere of carbon dioxide increases the oxygen pressure for 50% oxyhaemoglobin by about the same relatively small amount, namely, 1½ times.

When aquatic animals with haemoglobin in their blood are in water containing little dissolved oxygen there must at each temperature be a limiting low concentration of oxygen in the water below which the pressure gradient through the body wall is insufficient to maintain the haemoglobin in the oxygenated state, since oxygen is continuously being removed from the respiratory pigment by metabolic activities. It is possible to make an approximate estimation of this limiting external oxygen pressure by confining translucent animals in stoppered glass vessels full of water, watching with a spectroscope for the disappearance of the two oxyhaemoglobin absorption bands and as soon as they vanish determining the oxygen content of the water. This was done with *Tubifex* sp., *Daphnia magna*, the larvae of *Chironomus plumosus* L. and the variety of *Planorbis corneus* lacking skin pigmentation. The animals were confined in stoppered glass tubes at 17° C. and the dissolved oxygen was determined by the syringe-pipette modification of the Winkler method (Fox & Wingfield, 1938). With *Tubifex* not more than a dozen individuals were used in each tube because the animals clump into a ball, which, if large, must have almost anaerobic conditions in its interior; the outer worms then pass oxygen inwards and lose their oxyhaemoglobin bands at a relatively high external oxygen pressure. With only a dozen *Tubifex*, however, a small loose ball is formed from which the worms disentangle themselves entirely at low oxygen pressures. With *Chironomus* and *Planorbis* the bands do not vanish simultaneously in all the individuals in a tube, owing, no doubt, to different individual metabolic rates; the oxygen was determined when the last bands disappeared, but it is clear that in many individuals the haemoglobin had given up its oxygen at a somewhat higher external oxygen pressure. For *Planorbis* the end-point was the disappearance of the bands in the extended foot and head; the bands go sooner in those parts of the body that are within the shell. Six to ten experiments were made with each kind of animal.

With *Chironomus*, *Planorbis* and *Daphnia* the average oxygen pressures in the water for the disappearance of oxyhaemoglobin in the blood were respectively 13, 25 and 28 mm. of mercury. These values correspond to the oxygen affinities of the three haemoglobins, that of *Chironomus* having the highest, of *Daphnia* the lowest affinity. Reference to Table 1 shows, however, that these oxygen pressures in the water are much higher than those for 50% oxyhaemoglobin in the blood and therefore higher still than the pressures for complete deoxygenation of the haemoglobins. In the case of *Chironomus* the external oxygen pressure of 13 mm. for deoxygenation of the haemoglobin in the animal's blood is seen to be 22 times the internal pressure of 0·6 mm. for 50% oxyhaemoglobin (provided that the haemoglobins of *Chironomus plumosus* and *C. riparius* are similar). For *Planorbis* the external oxygen pressure is between 8 and 9 times the internal pressure, for *Daphnia* it is between 5 and 6 times, assuming the carbon dioxide pressure in the bloods to be 7·6 mm. (i.e. 1%). We do not know what the internal pressure of carbon dioxide actually is; for *Chironomus* haemoglobin it is of no importance. With *Tubifex* the oxygen pressure in the water when the oxyhaemoglobin bands vanished was so low that it could not be determined, the Winkler method not being accurate below an oxygen concentration of *c.* 0·4 ml. per l. owing to the oxygen content of the reagents. At 17° C. this oxygen concentration of 0·4 ml. per l. corresponds to a pressure of 10 mm., and the value for the disappearance of oxyhaemoglobin inside *Tubifex* was in this neighbourhood.* Clearly the difference between external and internal oxygen pressures is less here (cf. Table 1) than for the other three forms studied, but it is still considerable. In reality, of course, the difference between outside and inside oxygen pressures is in all cases greater than those given above because we have been comparing the external oxygen pressure for complete deoxygenation of the haemoglobin in the blood with the internal pressure for only 50% deoxygenation. Moreover, as stated above, in many individuals of *Chironomus* and *Planorbis* the haemoglobin loses its oxygen at higher outside oxygen pressures than those measured.

Leitch (1917) had already found a considerable difference between the external oxygen pressure for the deoxygenation of the blood of *Chironomus* and *Planorbis* and the pressure for deoxygenation in the diluted blood *in vitro*, without, however, drawing

* Dausend (1931) concluded from his experiments on the oxygen intake of *Tubifex* with normal and with carboxyhaemoglobin in waters of decreasing oxygen content that the haemoglobin in the blood remains fully oxygenated down to an oxygen pressure in the water of about 12 mm. at 19° C.

any conclusion as to the oxygen gradient through the body wall. But it is clear that in these animals, and in *Daphnia*, there must be a steep oxygen gradient across the body wall. This implies that the mode of oxygenation of their blood and tissues is not a particularly efficient one. In *Tubifex* the gradient is less steep, owing perhaps to a thinner skin and relatively greater respiratory surface. The more or less steep gradient across the body wall of these aquatic animals means that if they use their haemoglobin to pick up oxygen from water containing little, but still appreciable quantities, of the gas they must have haemoglobin with a high oxygen affinity, as indeed they have. It is clear, as pointed out by Harnisch (1930), that no deduction can be made from the dissociation curve of the haemoglobin as to the lowest oxygen content of the water in which aerobic existence can be maintained.

In the case of *Spirographis* there is no need for a respiratory pigment with a high oxygen affinity since there is plenty of oxygen in the sea water in which the crown expands, nor could the chlorocruorin pick up and transport oxygen from water deficient in the gas after the animal withdraws into its tube, for then the blood circulation stops (Fox, 1933).

SUMMARY

1. A method is described for measuring the oxygen affinity of haemoglobin in small samples of whole or slightly diluted blood or coelomic fluid.

2. The oxygen affinity of the haemoglobins of the following invertebrate animals is high: *Chironomus riparius*, *Tubifex* sp., *Ceriodaphnia laticaudata*, *Thalassema neptuni*, *Arenicola marina*, *Planorbis corneus*, *Daphnia magna*. The oxygen pressure in millimetres of mercury for 50% oxyhaemoglobin at 17° C. in the absence of carbon dioxide varies, in the order of species just given, from 0·6 for *Chironomus* to 3·1 for *Daphnia*. This may be compared with 27 for human whole blood at 20° C. and *p*H 7·4.

3. A carbon dioxide concentration of 1% of an atmosphere has no measurable effect on the oxygen affinity of *Chironomus* haemoglobin. In the other invertebrates studied this concentration of carbon dioxide increases the oxygen affinity by about 1½ times, which is less than half its effect on human haemoglobin.

4. When the haemoglobins of *Tubifex*, *Chironomus*, *Daphnia* and *Planorbis* are becoming deoxygenated in the animal there is a much lower oxygen pressure in the blood than in the water outside the animal. This means a steep gradient of oxygen pressure across the body wall. Consequently, for the animal to pick up oxygen from water containing little, but still considerable, quantities of the gas, haemoglobin of high oxygen affinity is essential.

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APPENDIX

The percentage saturation of haemoglobin with oxygen found in each experiment is given in Table 2. The mean value of the several oxyhaemoglobin percentages entered in the table for each temperature and oxygen pressure, without or with carbon dioxide, gives a point on an oxygen dissociation curve. From the partial oxygen dissociation curves thus constructed the oxygen pressures for 50% oxyhaemoglobin were derived which are given in Table 1.

With the blood of the common earthworm, *Lumbricus terrestris* L., I was unable to get consistent results.

I am grateful to Professor J. Gray, F.R.S., for the hospitality of his laboratory, to Dr R. Hill for advice, to Dr J. B. Gardner of the British Oxygen Company for measuring the residual oxygen in my purified nitrogen, and to Dr D. P. Pielou for drawing the figures.

Table 2. *Experimental data: oxyhaemoglobin percentages at different temperatures and oxygen pressures, without and with carbon dioxide*

	No carbon dioxide			1 % carbon dioxide		
	Oxygen mm. Hg.	% oxyhaemoglobin		Oxygen mm. Hg	% oxyhaemoglobin	
		10° C.	17° C.		10° C.	17° C.
Chironomus	0·7	77, 66, 68	65, 55, 58	0·7	70, 74	62, 55, 57
Tubifex	0·7	66	60	—	—	—
	1·3	—	84	—	—	—
Ceriodaphnia	0·7	—	45, 46	0·7	—	38, 35
	1·3	—	80, 65	1·3	—	58, 65
Homo	0·7	45, 50	28, 35	2·5	34, 33	13, 13
	1·3	70, 69	52, 50	3·6	60, 69	43, 43
Thalassema	0·7	41, 43	28, 28	0·7	41, 33	28, 27
	1·6	72, 70	55, 54	1·6	54, 53	45, 44
Arenicola	0·7	17, 21	9, 10	1·9	41, 50	24, 25
	1·9	63, 68	60, 50	3·0	78	62
Planorbis	1·3	45	28	1·9	41, 40	33, 31
	1·9	61, 58	47, 47	3·0	72, 67	54, 53
Daphnia	1·3	39, 40	21, 24	3·0	50, 50	36, 36
	2·5	56, 51	46, 39	4·2	63, 63	44, 47
	3·0	68	48	—	—	—
	3·6	75, 73	56, 59	—	—	—

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